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(71) Applicant (for all designated States except US): DEVGEN N.V. [BE/BE]; Technologiepark 9, B-9052 Wondelgem (BE).

(72) Inventors; and
(75) Inventors/Applicants (for US only): PLAETINCK, Geert [BE/BE]; Devgen N.V., Technologiepark 9, B-9052 Wondelgem (BE). PLATTEEUW, Christ [BE/BE]; Devgen N.V., Technologiepark 9, B-9052 Wondelgem (BE). MORTIER, Katherine [BE/BE]; Devgen N.V., Technologiepark 9, B-9052 Wondelgem (BE). BOGAERT, Thierry [BE/BE]; Devgen N.V., Technologiepark 9, B-9052 Wondelgem (BE).

(74) Agent: BOULT WADE TENNANT; 27 Furnival Street, London EC4A 1PQ (GB).

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(54) Title: CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION

(57) Abstract

There is provided a method of identifying DNA responsible for conferring a particular phenotype in a cell which method comprises a) constructing a cDNA or genomic library of the DNA of said cell in a suitable vector in an orientation relative to a promoter(s) capable of initiating transcription of said cDNA or DNA to double stranded (ds) RNA upon binding of an appropriate transcription factor to said promoter(s), b) introducing said library into one or more of said cells comprising said transcription factor, and c) identifying and isolating a particular phenotype of said cell comprising said library and identifying the DNA or cDNA fragment from said library responsible for conferring said phenotype. Using this technique it is also possible to assign function to a known DNA sequence by a) identifying a homologue(s) of said DNA sequence in a cell, b) isolating the relevant DNA homologue(s) or a fragment thereof from said cell, c) cloning said homologue or fragment thereof into an appropriate vector in an orientation relative to a suitable promoter(s) capable of initiating transcription of dsRNA from said DNA homologue or fragment upon binding of an appropriate transcription factor to said promoter(s) and d) introducing said vector into said cell from step a) comprising said transcription factor.

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CHARACTERISATION OF GENE FUNCTION
USING DOUBLE STRANDED RNA INHIBITION

The present invention is concerned with
characterization or identification of gene function
using double stranded RNA inhibition (dsRNAi) and
methods of identifying DNA responsible for inducing a
specific phenotype in a cell and a method of assigning
function to known gene sequences.

It has recently been described in Nature Vol 391,
pp.806-811, February 98, that introducing double
stranded RNA into a cell results in potent and
specific interference with expression of endogenous
genes in the cell and which interference is
substantially more effective than providing either RNA
strand individually as proposed in antisense
technology. This specific reduction of the activity
of the gene was also found to occur in the nematode
worm Caenorhabditis elegans (*C. elegans*) when the RNA
was introduced into the genome or body cavity of the
worm.

The present inventors have utilized this
technique and applied it further to devise novel and
inventive methods of assigning functions to genes or
DNA fragments, which have been sequenced in various
projects, such as, for example, the human genome
project and which have yet to be accorded a particular
function and for use in identifying DNA responsible
for conferring a particular phenotype.

Therefore, according to a first aspect of the
present invention there is provided a method of
identifying DNA responsible for conferring a phenotype
in a cell which method comprises a) constructing a
cDNA or genomic library of the DNA of said cell in an
orientation relative to a promoter(s) capable of

promoting transcription of said cDNA or DNA to double
stranded (ds) RNA upon binding of an appropriate
transcription factor to said promoter(s), b)
introducing said library into one or more of said

5   cells comprising said transcription factor, and c)
identifying and isolating a desired phenotype of said
cell comprising said library and identifying the DNA
or cDNA fragment from said library responsible for
conferring said phenotype.

10   In a preferred embodiment of the invention the
library may be organised into hierarchical pools as
described in more detail in the examples provided,
prior to step b) such as to include, for example, gene
families.

15   According to a further aspect of the invention
there is also provided a method of assigning function
to a known DNA sequence which method comprises
a) identifying a homologue(s) of said DNA in a cell, b)
isolating the relevant DNA homologue(s) or a fragment

20   thereof from said cell, c) cloning said homologue or
fragment into an appropriate vector in an orientation
relative to a promoter(s) capable of promoting
transcription of dsRNA upon binding of an appropriate
transcription factor to said promoters, d) introducing

25   said vector into said cell from step a) comprising
said transcription factor, and e) identifying the
phenotype of said cell compared to wild type.

In each aspect of the invention, the nucleotide
or DNA sequence may either be provided in a sense and

30   an antisense orientation relative to a single promoter
which has the properties defined above, or
alternatively it may be provided between two identical
promoters. In both embodiments dsRNA is provided from
the transcription initiated from the promoter

35   following binding of its appropriate transcription

factor.

The cell according to the invention may be
derived from or contained in an organism. Where the
cell is contained within an organism, the organism may
5   be adapted to express the appropriate transcription
factor. The organism may be any of a plant, animal,
fungus or yeast but preferably may be the nematode
worm *C. elegans*, which may be any of a wild type, a
nuc-1 or pha-ts mutant of *C. elegans* or a combination
10   of said mutations. In an alternative embodiment the
DNA or cDNA library or the DNA homologue or fragment
thereof may, advantageously, be transfected or
transformed into a microorganism, such as a bacterial
or yeast cell, which may be fed to the organism, which
15   is preferably the nematode worm *C. elegans*. In this
embodiment of the invention the microorganism may be
adapted to express the appropriate transcription
factor. Preferably, the microorganism is *E. coli*.

In each aspect of the invention, the DNA library,
20   DNA homologue or DNA fragment may be constructed in a
suitable DNA vector which comprises a sequence of
nucleotides which encode said transcription factor.
Alternatively, said transcription factor is encoded by
a further vector. In an even further alternative, the
25   cell or organism may express or be adapted to express
said transcription factor. Preferably, any of the
vectors used in the method according to the invention
comprises a selectable marker which may be, for
example, a nucleotide sequence encoding sup-35 or a
30   fragment thereof. The nucleotide sequence may be
orientated relative to a promoter such that binding of
a transcription factor to the promoter initiates
transcription of the DNA into double stranded RNA.
Figure 10 illustrates the vectors and the orientation
35   of the DNA sequence which enable double stranded RNA

production in *C. elegans*. Thus in one embodiment the
DNA is located between two promoters on a vector
capable of expressing dsRNA upon binding of an
appropriate transcription factor to said promoters.

5   Alternatively, the vector comprises two copies of the
DNA sequence organised in a sense and antisense
orientation relative to the promoter and which marker
is selectable when contained in a pha-1 mutant *C.
elegans*. Preferably, the promoters are any of T7, T3

10   or SP6 promoters and the transcription factor
comprises the appropriate polymerase.

       Preferably, the selectable marker comprises a
nucleotide sequence capable of inhibiting or
preventing expression of a gene in said cell and which

15   gene is responsible for conferring a known phenotype.
This nucleotide sequence may be part of or identical
to said gene conferring said phenotype, and which
nucleotide sequence is itself oriented relative to a
suitable promoter(s) capable of initiating

20   transcription of double stranded RNA upon binding of
an appropriate transcription factor to said
promoter(s). Alternatively, the nucleotide sequence
may be a part of or identical to said gene sequence
conferring said phenotype, and which nucleotide

25   sequence is such as to permit integration of said
suitable or further vector by homologous recombination
in the genome of said cell and following said
integration said nucleotide sequence is capable of
inhibiting expression of said gene sequence conferring

30   said phenotype. In this embodiment said nucleotide
sequence comprises stop codons sufficient to prevent
translation of said nucleotide sequence following its
integration into said genome.

       Compounds can, advantageously, in said method be

35   added to said cell or organism for the purposes of

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screening for desired phenotypes, such as for example,
resistance or sensitivity to the compound when
compared to wild type. The promoters are preferably
inducible. The transcription factor may in some
embodiments be phage derived, such as for example, a
T7 polymerase driven by a phage promoter. However,
when *C. elegans* is utilised a worm specific or tissue
specific promoter can be used, such as for example,
let858, SERCA, UL6, myo-2 or myo-3. Preferably, the *E.
coil* strain is an RNAaseIII and even more preferably
an Rnase negative strain.

A further aspect of the present invention
provides a method of generating a transgenic non-human
organism comprising an exogenous transcription factor
and a transgene comprising a promoter operably linked
to DNA fragment which is expressed upon binding of
said transcription factor thereto, the method
comprising a) providing a first transgenic organism
comprising a first construct incorporating DNA
encoding an exogenous transcription factor and a
second transgenic organism comprising a second
construct including at least one promoter operably
linked to a desired DNA sequence which is expressed
upon binding of the transcription factor of said first
transgenic organism thereto b) crossing said first and
second transgenic organisms and selecting offspring
expressing said desired DNA sequence. In one
embodiment said first and second transgenic organisms
are generated by transforming said first and second
constructs into respective microorganisms for
subsequent feeding to the respective organism.
Preferably, said second construct comprises said
desired DNA sequence in an orientation relative to
said promoter so as to be capable of initiating
transcription of said DNA to dsRNA upon binding of

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said transcription factor thereto. In this embodiment
said second construct comprises two promoters flanking
said desired DNA sequence which promoters can initiate
transcription of said DNA sequence to dsRNA upon
5   binding of said transcription factor to said
promoters. Alternatively, said DNA sequence is
provided in a sense and an antisense orientation
relative to said promoter so as to produce dsRNA upon
binding of the transcription factor to the promoters..
10   In each of these embodiments the first and/or second
constructs may preferably be provided with a reporter
gene operably linked to a promoter which is capable of
initiating transcription of said reporter upon binding
of said transcription factor thereto. Preferably, the
15   reporter gene encodes any of Luciferase, Green
Fluorescent protein, β galactosidase or β-lactamase.

The present invention also includes a method of
validating clones identified in yeast two hybrid
vector experiments which experiments are well known to
20   those skilled in the art and which experiments were
first proposed by Chien *et al.* (1991) to detect
protein - protein interactions. The method according
to the invention comprises providing a construct
including the DNA encoding a protein identified in a
25   two hybrid vector experiment, which construct is such
that said DNA is provided in an orientation relative
to one or more promoters capable of promoting
transcription of said DNA to double stranded RNA upon
binding of an appropriate transcription factor to said
30   promoters, transforming a cell, such as a bacterial
cell or alternatively transforming an organism
comprising said transcription factor with said
constructs and identifying a phenotypic change in said
cell or organism, which may be *C. elegans* or the like,
35   compared to wild type. Preferably, the transcription

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factor is inducible in the cell or organism. Once
again the DNA sequence may be located between two
promoters or in both a sense and antisense orientation
relative to a single promoter, as described above.
5   Preferably, the promoter is a phage polymerase
promoter and said transcription factor is a RNA
polymerase, and preferably T7 polymerases. Also
encompassed with the scope of the present invention
are vectors used to transform said cells or organisms
10   and the cells or organisms themselves.

        In a further aspect of the present invention
there is provided a method of alleviating pest
infestation of plants, which method comprises a)
identifying a DNA sequence from said pest which is
15   critical either for its survival, growth,
proliferation or reproduction, b) cloning said
sequence from step a) or a fragment thereof in a
suitable vector relative to one or more promoters
capable of transcribing said sequence to RNA or dsRNA
20   upon binding of an appropriate transcription factor to
said promoters, and c) introducing said vector into
the plant.

        Thus, advantageously, the method according to the
invention provides a particularly selective mechanism
25   for alleviating pest infestation, and in some cases
parasitic infestation of plants, such that when the
pest feeds on the plant it will digest the expressed
dsRNA in the plant thus inhibiting the expression of
the DNA within the pest which is critical for its
30   growth, survival, proliferation or reproduction. In a
preferred embodiment, the pest may be any of
Tylenchulus ssp. Radopholus ssp., Rhadinaphelenchus
ssp., Heterodera ssp., Rotylenchulus ssp.,
Pratylenchus ssp., Belonolaimus ssp., Canjanus ssp.,
35   Meloidogyne ssp., Globodera ssp., Nacobbus ssp.,

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Ditylenchus ssp., Aphelenchoides ssp., Hirschmenniella
ssp., Anguina ssp., Hoplolaimus ssp., Heliotylenchus
ssp., Criconemellassp., Xiphinemassp., Longidorus
ssp., Trichodorus ssp., Paratrichodorus ssp.,
Aphelenchs ssp. The DNA sequence or fragment thereof
according to this aspect of the invention may be
cloned between two tissue specific promoters, such as
two root specific promoters.

A further aspect of the invention concerns the
vector used in each of the methods of the invention
for constructing said library, which vector comprises
two identical promoters oriented such that they are
capable of initiating transcription of DNA sequence
located between said promoters to dsRNA upon binding
of an appropriate transcription factor to said
promoters. The DNA sequence may, for example, include
a multiple cloning site. Preferably, the expression
vector comprises a nucleotide sequence encoding a
selectable marker. In one embodiment the nucleotide
sequence encoding said selectable marker is located
between two identical promoters oriented such that
they are capable of initiating transcription of DNA
located between said promoters to double stranded RNA
upon binding of an appropriate transcription factor to
said promoters. Preferably, the selectable marker
comprises a nucleotide sequence encoding sup-35, for
introduction into C. elegans having a pha-1 mutation.

Preferably, the transcription factor comprises
either a phage polymerase which binds to its
corresponding promoter or a C. elegans specific
promoter and even more preferably T7 polymerase.
Preferably, the vector includes a multiple cloning
site between said identical promoters.

In a further aspect of the invention there is
provided an expression vector for expressing an

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appropriate transcription factor for use in a method
according to the invention which vector comprises a
sequence of nucleotides encoding said transcription
factor operably linked to suitable expression control
5   sequences. Preferably, the expression control
sequences include promoters which are inducible,
constitutive, general or tissue specific promoters, or
combinations thereof. Preferably, the transcription
factor comprises a phage polymerase, and preferably
10   T7, T3 or SP6, RNA polymerase.

       A further aspect of the invention provides a
selection system for identifying transformation of a
cell or organism with a vector according to the
invention which system comprises a vector according to
15   the invention wherein said selectable marker comprises
a nucleotide sequence capable of inhibiting or
preventing expression of a gene in said cell or
organism which gene is responsible for conferring a
known phenotype. Preferably said nucleotide sequence
20   corresponds to a part of or is identical to said gene
conferring said known phenotype, and which nucleotide
sequence is itself located between two identical
promoters capable of initiating transcription of
double stranded RNA upon binding of an appropriate
25   transcription factor thereto. Alternatively, the
nucleotide sequence comprises a nucleotide sequence
which is a part of or identical to said gene sequence
which confers a known phenotype on said cell or
organism, and which is such that following integration
30   of said vector by homologous recombination in the
chromosome of said cell or organism said sequence
inhibits expression of said gene sequence conferring
said known phenotype. Preferably, according to this
embodiment the
35   nucleotide sequence comprises stop codons sufficient

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to prevent translation of the nucleotide sequence
following integration into said chromosome.
Preferably, the known gene sequence comprises a sup-35
gene or a fragment thereof which is selectable by
5   identifying offspring growing at a temperature above
25°C following introduction in a pha-1 et123ts mutant
C. elegans worm.

In a further aspect of the invention provides
said known gene sequence comprises a sup-35 gene or a
10   fragment thereof which is selectable by identifying
offspring growing at a temperature above 25°C
following introduction of said vector in a pha-1
et123ts mutant C. elegans worm. An even further
aspect comprises a method of assigning function to a
15   DNA sequence of a multicellular organism which method
comprises a) providing i) a construct comprising said
DNA fragment cloned between two promoters capable of
promoting transcription in said multicellular
organism, in a multicellular organism capable of
20   initiating transcription from said promoter; b)
identifying the phenotype of said multicellular
organism compared to wild type.

The present invention may be more clearly
understood by the following examples which are purely
25   exemplary with reference to the accompanying figures,
wherein:

Figure 1   is a nucleotide sequence of plasmid PGN1 in
           accordance with the present invention.

30

Figure 2   is a nucleotide sequence of plasmid PGN100
           in accordance with the present invention.

Figure 3   is a schematic representation of the vectors
           used and the transformation regime used in
35   the methods according to the present

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invention.

Figure 4   is an illustration of an expression vector
            used in accordance with the invention.

Figure 5   is a schematic illustration of the T7 RNA
            polymerase expression vectors used for
            transforming *C. elegans*.

Figure 6   is an illustration of plasmid PGN1.

Figure 7   is a diagrammatic representation of an
            enhanced vector for dsRNA inhibition
            encoding sup-35 dsRNA.

Figure 8   is an illustration of a vector for
            integration into the genome of *C. elegans*.

Figure 9   is an illustration of the position of a DNA
            sequence(s) relative to a suitable promoter
            to initiate expression of dsRNA from the DNA
            sequence(s).

Figure 10   is a representation of plasmid pGN108.

Figure 11   is a representation of plasmid pGN105.

Figure 12   is a representation of plasmid pGN400.

Figure 13   is a representation of plasmid pGN401.

Figure 14   is a representation of plasmid pGN110.

Figure 15   is a representation of plasmid pAS2 with
            forward and reverse T7/T3/SP6 promoters.

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Figure 16 is a representation of plasmid pGAD424 with
forward and reverse T7/T3/SP6 promoters.

Figure 17 is a representation of plasmid pAS2-cyh2-
HA+, both T7-final.

Figure 18 is a representation of plasmid pGAD424-
without-FULL-ICE-BOTH-T7.

Figure 19 (a) is a representation of plasmid pGN205
and(b) is a representation of plasmid
pGN207.

**Example A: Construction of an ordered and
hierarchical pooled cDNA library and applications
thereof.**

A random ordered and pooled library:
The vector is an *E. coli* vector harboring two T7
promoters, with a multiple cloning site (MCS) in
between. The two promoters are orientated towards
each other, and towards the MCS. In the presence of
T7 RNA polymerase, expressed in *E. coli, C. elegans* or
any other organism, RNA will be produced, starting
from the two T7 promoters. As these are oriented in
the opposite sense, both strands of RNA will be
produced from the DNA inserted (cloned) into the MCS
in between the two promoters which results in the
generation of double stranded RNA (dsRNA) upon binding
of the T7 RNA polymerase thereto.
A *C. elegans* cDNA library is constructed in the
MCS using standard molecular biological techniques.
The library is transformed into *E. coli*, and the
resulting *E. coli* are grown in culture and stored in
96 multi-well plates. At this stage, plasmid DNA can

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be isolated and stored in 96-multi-well plates
corresponding to those of the *E. coli* colonies.
Approximately 100,000 colonies are scored. In this
way, the library will harbor approximately 5 times the

5   total expressed cDNA variation of *C. elegans*, which
gives the opportunity for low expressed sequences to
be present in the library. This will result in
approximately 1041 96-well plates. The plates are
hierarchical pooled as necessary. For the present

10   pooling of the clones is arranged in a range of 10 to
100. If the hierarchical pooling is per 8 or 12
(numbers are more convenient as 96-well plates have a
8 to 12 grid), this will result in approximately 87
multi-well plates and approximately 8352 wells. If

15   hierarchical pooling is per 96 wells, which is a full
plate, this results in approximately 11 plates and
approximately 1041 wells. At any stage of the
hierarchical pooling, plasmid DNA can be isolated,
which would be less elaborate as less plates are used,

20   but will result in a loss of complexity although this
should not be the case in the pooling per 12. The
pooling of the DNA can also be carried out with the
original DNA.

       The experiments below describe how the
25   hierarchical pooling should be performed, both for the
DNA and for the *E. coli* library.

       **An ordered library for RNAi technology, harboring
       every gene of the C. elegans genome, with**
30   **applications thereof**

       As the genome-sequencing project is coming to an
end, this information can be used in the application
of T7 RNA inhibition technology. Every gene of the *C.*
35   *elegans* genome can be cloned using PCR technology. In

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preference, exons will be cloned with a minimal length
of 500 bp. If the exons are too small, smaller
fragments will be isolated with PCR, or even parts of
introns and neighboring exons will be isolated with
5   PCR technology so that at least a sufficient part of
the translated region of the gene is cloned. For
this, at least 17000 PCR reactions need to be
performed. This collection of PCR products will be
cloned in a T7 vector as described (two T7 promoters
10   oriented towards each other with a multiple cloning
site in between). Every PCR product is cloned
independently, or can be used to generate a random
library, analogous to the described cDNA library. If
every PCR product is cloned individually, the
15   resulting bacteria and plasmid DNA can be pooled in
various ways. Firstly, this collection of individually
cloned PCR products in the T7 RNAi vector can be
pooled randomly, as described in the random library.
This pooling can also be done in a more rational way.
20   For instance, the genes of the C. elegans genome can
be analyzed using bioinformatic tools (in silico
biology). Various genes of the
genome will belong to a gene family, or will have
homologues in the genome. These members of the gene
25   family will be pooled, or the members, being
homologues will be pooled. In this way the total
number of about 17000 clones is reduced to a more
useable quantity. This library can be used to screen
for phenotypes in the methods according to the
30   invention. The resulting phenotype gives a functional
description to the gene or gene family or gene
homologues of the C. elegans genome. As the library
consists of a part of every gene in the genome, this
method enables description of the full genome in
35   functional-phenotypic terms. For this the double

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stranded RNA (dsRNA) needs to be introduced in the
worm. This introduction of clones alone, or pooled
clones, being random pooling or rational pooling can
be achieved in several ways as described.

5

**Example of a vector for the expression of double
stranded RNAi**

Any vector containing a T7 promoter may be used,
10   and which contains a multiple cloning site (there are
many commercially available). Primers containing the
T7 promoter and a primer with the reverse
complementary strand, both with the appropriate ends
are designed. These primers can be hybridized, and if
15   well designed, cloned in the vector of choice. The
minimal sequence for a T7 promoter is
TAATACGACTCACTATAGGGCGA. Although any vector can be
used for the construction of a T7 expression vector
there follows an example of how to achieve this with
20   the vector pGEM-3zf(-).
        - Vector pGEM-3zf(+) (PROMEGA) was digested with
        HindIII and SalI
        - Primers oGN1 and oGN2 were mixed together at a final
        concentration of 1 μg/30 μl boiled and cooled slowly
25   to room temperature.
        - The primer was ligated into the vector using
        standard ligation procedures. The resulting vector is
        pGN1 (shown in Figure 1) and contains two T7 promoters
        oriented towards each other, and harbors a multiple
30   cloning site in between.
        Sequences of oGN1 and oGN2 are:
        - oGN1: AGC TGT AAT ACG ACT CAC TAT AGG GCG AGA AGC TT
        - oGN2: TCG AAA GCT TCT CGC ATA ATA GTG AGT CGT ATT AC

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Example of the construction of a library

RNA may be isolated from every organism that is
sensitive to RNAi. In general the isolated RNA is then
5   copied into double stranded cDNA, and subsequently
prepared in suitable vectors for cloning. Several
procedures exist and molecular biology kits can be
purchased from various firms including promega,
clontech, boehringer Mannheim, BRL, etc which enable:
10

- isolation of RNA,
- eventually polyA RNA can be isolated (several
techniques and kits available)
- first strand synthesis with AMV reverse
15   transcriptase, random hexameric primers and/or
oligo (dT) primer
- second strand synthesis with Rnase H, DNA
PolymeraseI,
- flush ends with T4 DNA Polymerase
20   - addition of an adaptor with T4 DNA ligase.
- eventually treatment with T4 polynucleotide Kinase
- cloning of the cDNA into the vector.

The resulting ligation mixture can be considered
25   as the cDNA library. The ligation contains all cDNA of
the procedure ligated into the vector of interest.
To order the library, the ligation needs to be
transformed into *E. coli* strains.

30   **Application of this E. coli or DNA library**

T7 RNA producing strain:
- a standard strain is BL21 (DE3): F-ompT[lon]hsds(r-
m-; and *E. coli* B strain) λ (DE3). Eventually
35   variants of BL21 (DE3) can be used, although BL21

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(DE3)pLysS is used.

- any other *E. coli* strain which produces the T7 RNA
polymerase, which may be available needs to be
constructed. This can be generated easily using a
phage, which is commercially available, in this case,
the λCE6 vector (provided by Promega) is used. Almost
every *E. coli* strain can be transfected with this
phage and will produce T7 RNA polymerase.

- a RNAseIII mutant *E. coli*:
Various strains are in principle available, we chose
in a first experiment to use strain AB301-105: rna-19,
suc-11, bio-3, gdhA2, his95, rnc-105, relA1, spoT1,
metB1. (Kinder *et al.* 1973 Mol. Gen. Genet 126:53),
but other strains may suit better. This strain is
infected with λCE6 and so a T7 producing variant will
be constructed.

Wild type *C. elegans* worms can be grown on the
bacteria pools. The bacteria is expressing the T7 RNA
polymerase. This results in large quantities of dsRNA
in the gut of the *C. elegans*, which will diffuse in
the organism and results in the inhibition of
expression. This library can now be used for the
screening of several phenotypes. This technique has
the advantage that it is a much faster to detect
relevant genes in certain pathways, than the known *C.
elegans* technology. Moreover, if an interesting
phenotype is found, the responsible gene can be cloned
easily.

Using the hierarchical pooling one can easily find in
a second screen the relevant clone of the pool. The
inserted DNA of this clone can then be sequenced.
This experiment results in genetic and biochemical
DATA in one step.

Wild type *C. elegans* strains can be combined with
compounds to screen for phenotype, drug resistance and

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or drug sensibility. The *C. elegans* strain can be a
mutant strain, screening for an enhanced phenotype,
reduced phenotype, or a new phenotype. The *C. elegans*
strain can be a mutant strain, and the library screen
can be combined with compounds. So one can screen for
drug resistance, drug sensibility, enhanced phenotype,
reduced phenotype, or a new phenotype. The *E. coli*
strain may be any T7 RNA polymerase expressing strain,
like BL21 (DE3), for example, but the formation of
double strand RNA may be enhanced by using a special
E. coli strain that is RNAseIII negative. RNAseIII
recognizes specific loops in dsRNA. Eventually, an *E. coli* strain can be used that is deleted in RNAses
other than RNAseIII or an *E. coli* can be used that is
deleted in one or more RNAses. The expression of the
T7 RNA polymerase in most known *E. coli* strains and
constructs which are available to generate T7 RNA
polymerase producing *E. coli* strains, generally
comprise an inducible promoter. In this way the
production of the T7 RNA polymerase is regulated, and
thus the production of the dsRNA. Advantageously,
this feature can be used to "pulse" feed the *C.
elegans* worms at specific stages of growth. The worms
are grown on the non-induced *E. coli* strains. When
the worm has reached the stage of interest, the T7 RNA
production in the bacteria is induced. This allows
the studying of the function of any gene at any point
in the life cycle of the animal.

**Screening the library for homologues of putative
interesting human genes, and assign function to
these genes**

Hundreds of genes have been isolated in various
projects, being genomic projects, differential

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expressed arrays, hybridization studies, etc. The described cDNA library can provide a way to validate and or assign function to these genes in a fast and efficient manner. First of all the worm homologue or
5   homologues or the genes need to be identified by bioinformatic tools (in silico biology). PCR primers are developed and the cDNA fragment is isolated using PCR technology. PCR can be performed on the hierarchical pools. The positive pool or individual
10 wells harboring the bacteria that has the appropriate cDNA is fed to *C. elegans* and the phenotype is scored.

PCR can be performed on cDNA isolated from *C. elegans*. The resulting DNA can be cloned in the T7 vector and transformed in the dsRNA producing *E. coli*
15 on which the *C. elegans* worms are then fed. Depending on which way is faster and more reliable a choice needs to be made.

If the gene belongs to a gene family, the worm may need to be fed on a mixture of bacteria. Each of
20 them harboring a part of the member of the gene family. *E. coli* strains, growth conditions, combinations with compounds can be performed as described above.

If the library rational is used, in which all the
25 genes of *C. elegans* are cloned in a organized and structured way, the *C. elegans* homologue and eventually the other homologues, orthologues, and members of the gene family can be traced back easily in the library using a silico biology. No PCR is
30 involved in this step, and the bacteria and or DNA can be isolated on which the worm will be grown.

Examples

35   The idea of the series of experiments was to test

- 20 -

both the RNAi vector and the various *E. coli* strains
that were constructed.

 1) Construction of a test plasmid

5 Any cDNA that gives a clear phenotype in the worm
when knocked-out, or used in a RNAi experiment can be
used. It is known that unc-22 is a good candidate,
but may other genes are possible. We opted for a
sensitive system that can be used at a later stage.

10 The system was tested with sup-35 in a pha-1
background. Exon 5 of the sup-35 was isolated by PCR
and cloned in the T7 promoter vector pGN1. The
resulting vector was designated pGN2. pha-1 (e2123)
mutant worms cannot produce offspring at temperatures

15 higher than 25°C. This is due to a developmental
problem in embryogenesis. When sup-35 is knocked-out,
or inhibited in this strain, offspring may grow at
this temperature. Combination of pha-1 mutant worms
and sup-35 RNAi is a good system to validate the

20 various options.

 2) Testing the RNAi using an *E. coli* strain
that produces dsRNA.

- pGN2 was introduced in *E. coli* strain BL21(DE3) and

25 T7 RNA polymerase was induced with IPTG. *C. elegans*
worms (pha-1 (e2123)) were inoculated on this
bacteria, and grown at the restricted temperature of
25°C. As this mutant is an embryonic mutant at this
temperature, no offspring will be observed. If the

30 sup-35 gene is efficiently inhibited by the dsRNA
present in the *E. coli*, offspring will be observed.
- pGN2 was introduced in *E. coli* strain AB301-105(DE3)
and T7 RNA polymerase was induced with IPTG. *C.
elegans* worms (pha-1 (e2123)) were inoculated on this

35 bacteria, and grown at the restricted temperature of

- 21 -

25°C. As this mutant is an embryonic mutant at this
temperature, no offspring will be observed. If the
sup-35 gene is efficiently inhibited by the dsRNA
present in the *E. coli*, offspring will be observed.

5

3)   Improving the worm strain for better uptake
of dsRNA.

Before plating the pha-1 *C. elegans* on the *E.
coli* strain that produce the double stranded sup-35
RNA. The worm was mutagenised with EMS (Methane
sulfonic Acid Ethyl). The offspring of this
mutagenised worm is then plated on the bacteria. The
worm that feed on this bacteria give larger offspring
which has a mutation that results in an improvement of
dsRNA uptake, and can be used for further experiments.

**Stable integration of the dsRNA producing vector
into the genome of the T7 RNA polymerase
producing worm**

An *E. coli* vector can be constructed harboring
the following features; Two T7 promoters directed
towards each other, with a restriction site or a
multiple cloning site in between. Furthermore, the
vector may contain the *C. elegans sup35* genomic DNA,
engineered in such a way that it contains several
stopcodons at various intervals, so that no full
length protein can be expressed form the *sup35* genomic
DNA fragment as illustrated in Figure 8. Any cDNA or
cDNA fragment can be cloned in the multiple cloning
site between the two T7 promoters. When this vector
is introduced in a *C. elegans* strain which expresses
T7 RNA polymerase, the cDNA or DNA fragment cloned
between the two T7 promoters will be transcribed,
generating dsRNA from the cloned fragment.

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The vector is designed to be used in pha-1 (e2123) mutant worms expressing T7 RNA polymerase. The expression of the T7 RNA polymerase may be constitutive or regulated, general or tissue specific.

5   These pha-1 (e2123) worms cannot produce offspring at temperatures higher than 25°C, which is due to a development problem in embryogenesis. When sup-35 is inhibited or knocked-out in this stain, offspring may grow at this temperature.

10   When the vector is introduced in the worm, the vector may integrate by homologous recombination (Campbell-like integration). It has been shown that homologous recombination occurs in C. elegans, although at low frequencies (Plasterk and Groenen,

15   EMBO J. 11:287-290, 1992). Homologous recombination at the sup35 gene will result in a knock-out of the gene as the two resulting sup-35 genes will harbor the stopcodons. The resulting worm, and its offspring, if this recombination happens in the eggs, will have a

20   copy of the vector integrated in the genome. This can be selected as only the worms for which the sup-35 has been knocked-out will have offspring at temperatures higher than 25°C. Furthermore, the resulting worm will stably produce double stranded RNA from the DNA

25   fragment cloned between the two T7 promoters. This worm can now be considered as a stable transgenic worm strain with a reduction of function of the gene, from which a fragment has been cloned between the two T7 promoters.

30   The DNA may be delivered to the worm by several techniques, including injection, ballistic transformation, soaking in the DNA solution, feeding with bacteria. New and other methods that increase the transformation efficiencies can be considered.

35   The target C. elegans strain may in addition,

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have other mutations than the pha-1 (e2123) mutation,
and may express other genes than T7 RNA polymerase.

EXAMPLE B: a Yeast two-hybrid-RNAi vector

5

A yeast two hybrid vector can be constructed
harboring the two T7 promoters. The vectors can be
designed to replicate both in yeast and in *E. coil*.
In general cDNA libraries for the yeast two hybrid

10   system are made in the Gal4 or LexA vectors. The
library is constructed in vectors having the
activation domain of one of these genes. A vector can
be constructed that can still perform in the
yeast two hybrid screen but which also contains two T7

15   promoters orientated towards each other, with a
cloning site therein between. The order of the
sequences in the plasmid will then be "plasmid
backbone, (GAL4-T7), MCS, T7, backbone". A *C. elegans*
cDNA library constructed in this vector can be used as

20   a standard yeast two hybrid library in an experiment
to isolate interacting proteins with a given protein.
Once a clone is isolated, the plasmid can be
introduced in an *E. coil* strain expressing the
T7 RNA polymerase, and hence will produce dsRNA of the

25   cloned fragment. The bacteria producing this dsRNA
can be fed to the worm and phenotypes can be scored.
As in the previous example, this validation procedure
for a newly isolated yeast two hybrid clone is
remarkably shorter than the standard procedure, which

30   requires PCR and/or cloning steps, RNA experiments
and/or knock-out experiments. In most cases isolated
clones are sequenced first, and on the basis of the
sequence, a decision is made to continue with further
experiments. In the present invention every isolated

35   clone can easily be introduced into the appropriate *E.*

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coli and fed to the worm. Validation is then performed
by phenotype analysis.

To apply this procedure a yeast two hybrid was
performed using a known gene as bait and the newly
constructed library as the target. Proteins coded by
the clones in the target that interact with the bait
protein, will result in positive yeast clones
expressing the reporter molecule such as can be
observed by LacZ staining with X-gal. The plasmid
coding for the target protein is isolated directly
from the yeast strain and introduced in *E. coli*. The
E. coli is T7 RNA polymerase producing *E. coli*. In
this case, double stranded RNA is produced from the
DNA cloned in the multiple cloning site of the vector.
When this dsRNA is fed to the worm using the methods
described previously, the gene has inhibited in the
worm, resulting in a particular phenotype.
- This yeast two hybrid vector can advantageously be
used to construct an ordered and hierarchically pooled
library as described in the previous example.
- A yeast strain can also be constructed that
conditionally produces T7 RNA polymerase. After yeast
two hybrid experiments, the expression of the T7
polymerase could be induced, resulting in the
production of dsRNA in the yeast cell. Consequently
the yeast could be fed to the worm. Evidence is
available showing that the *C. elegans* worms can feed
on yeast.

**Construction of a T7 RNA polymerase producing
strain, and applications thereof**

A *C. elegans* strain can be constructed that
expresses T7 RNA polymerase. The expression can be
general and constitutive, but could also be regulated

- 25 -

under a tissue specific promoter, an inducible
promoter, or a temporal promoter or a promoter that
harbors one of these characteristics or combination of
characteristics. DNA can be introduced in this *C.*
5   *elegans* strain. This is done either by injection, by
shooting with particles, by electroporation or as
aforementioned by feeding. If the DNA is a plasmid as
described in the previous examples, i.e. a plasmid
harboring a cloned cDNA fragment or a PCR fragment
10   between two flanking T7 promoters, then dsRNA of this
cDNA or PCR fragment is formed in the cell or whole
organism resulting in down regulation of the
corresponding gene. The introduced DNA can have an
efficient transient down regulation. The introduced
15   DNA can form an extrachromosomal array, which array
might result in a more catalytic knock-out or
reduction of function phenotype. The plasmid might
also integrate into the genome of the organism,
resulting in the same catalytic knock out or reduction
20   of function phenotype, but which is stably
transmittable.
       - Plasmid DNA harboring a cDNA or a part of a cDNA or
an EST or an PCR fragment of *C. elegans* cloned between
two T7 promoters as described in Examples A) and B)
25   can be introduced in the T7 RNA polymerase worm, by
standard techniques. Phenotypes can be analysed -DNA
from an ordered and pooled library as in Example A)
can be introduced in the T7 RNA polymerase worm, by
standard techniques (injection, shooting). Phenotypes
30   can be analysed. With the hierarchical pool, the
original clone can be found easily.
       - The same procedure can be performed with a mutant
worm expressing the T7 RNA polymerase. Screening for
enhanced, reduced or new phenotypes.
35   - The procedure can be used to enable screening of

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compounds. Screening with either a wild-type strain or
a mutant strain for enhanced or new phenotypes.
- The DNA could be introduced in the worm by new
methods. One of which is the delivery of DNA by *E.*
5   *coli*. In this case the hierarchical pooled library is
fed to the animal. To prevent digestion of the *E. coli*
DNA in the gut of the nematode, preferentially a DNAse
deficient *C. elegans* will be used, such as nuc-1
(e1392). This procedure would be one of the most
10   interesting as it would be independent of
transformation efficiencies of other techniques, and
generally faster and less labourious.

        2) Putative enhancements of the method.
15   - A vector is designed, so that it harbors the sup-35
cDNA or a part of this cDNA, cloned in between two T7
promoters. The rest of the vector is as described in
Examples A) and B). This vector can be introduced into
a pha-1ts mutant *C. elegans*. A temperature selection
20   system exists in this case and only those worms which
have taken up the DNA and express the double stranded
sup-35 RNA will survive at restricted temperatures.
The hierarchical pooled library can be delivered by
any method described above.
25   - The vector can be used to construct a library that
is introduced in a T7 RNA polymerase expressing *E.*
coli. In this case we have an analogous screening as
in part A) with an additional screening for worms where
the dsRNA of sup-35 is active.
30   - The DNA and or dsRNA of sup-35 could be delivered on
a different plasmid. For the feeding, both DNA feeding
(Example C) or dsRNA feeding Example A) and B), this
means that the two plasmids could be present in one
bacterium, or that the worm is fed on a mixture of
35   bacteria, one of which harbors the sup-35 construct.

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Example of the construction of a T7 RNA producing
C. elegans

To produce T7 RNA polymerase in the worm, several
5   possibilities are possible. The T7 polymerase can be
expressed under various promoters, being inducible
promoters, constitutive promoters, general promoters
and tissue (cell) specific promoters, or combinations
of those. Examples of these promoters are the
10   heatshock promoter hsp-16, the gut promoter ges 1, the
promoter from cet858, but also the promoter of dpy 7
and the promoter element GATA1. In this example the T7
RNA polymerase is expressed under the control of the
hsp-16 promoter that is available in the pPD49.78
15   vector. The T7 RNA polymerase is isolated as a PCR
product using the primers of GN3 an GN4.
       The resulting PCR product is digested with NheI
and NcoI, as is the vector in which we want to clone,
being the Fire vector pPD49.78. The resulting vector
20   is pGN100 illustrated in Figure 2 oGN3: CAT GGC AGG
ATG AAC ACG ATT AAC ATC GC oGN4: ATG GCC CCA TGG TTA
CGG GAA CGC GAA GTC CG pGN100 is included.
       The vector is introduced into the worm using
standard techniques, such as micro injection, for
25   example.

       The following strains were then constructed:

       -Wild-type (pGN100)
30   -nuc-1 (e1392) (pGN100)
       -pha-1 (e2123) (pGN100)
       -pha-1; nuc-1   (pGN100)

       All of these strains are able to produce T7 RNA
35   polymerase when temperature induced or alternatively

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by metals such as application of heavy cadmium or
mercury. The procedure for temperature induction is to
shift the animal to a temperature of 30-33°C for at
least one hour, then the animal can be shifted back to

5   standard

temperatures (15-25°C).

The wild type strain producing T7 RNA polymerase
can be used for the production of any RNA in the worm.
More specifically, the plasmids from the described

10   libraries can be introduced in these worms, and

phenotypes can be scored.

The nuc-1 mutant worm will be used to introduce
DNA via bacteria on which the worm feed. As the nuc-1
worm does not digest the DNA, the plasmid DNA can

15   cross the gut wall. If taken up by the cells that

produce the T7 RNA polymerase, dsRNA will be produced
thus inhibiting the gene from which the RNA was
transcribed.

The pha-1 mutant strain that produced T7 RNA

20   polymerase can be used to enhance the procedures as

described above. DNA can be introduced by shooting,
micro injection or feeding. More specifically this
strain can be used for the vectors that produce dsRNA
from sup-35 and from the gene of interest, the latter

25   can be a PCR product, a cDNA, or a library as

described.

The pha-1; nuc-1 mutant producing T7 RNA
polymerase can be used for the bacterial delivery of
the DNA. DNA will preferentially be the plasmid that

30   produce dsRNA from both sup-35 and the gene of

interest. The worm strain will preferentially produce
the T7 RNA polymerase in the gut. Delivery will
preferentially happen by feeding the worm on bacteria
harboring the plasmid.

35

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Application of the RNAi technology in plants

Nematodes are responsible a large part of the
damage inflicted on plants and more particularly to
plants used in the agricultural industry. The RNAi
procedures according to the invention can be applied
to plants to prevent these parasitic nematodes from
feeding longer. In a first step, a DNA fragment is
isolated from the parasitic plant nematode that is
critical for the animals survival or growth, or to
feed or to proliferate. Any gene from which the
expression is essential is suitable for this purpose.

A part of this gene, an exon or cDNA is cloned.
This DNA fragment can be cloned under the influence of
a tissue specific promoter preferably a root specific
promoter even more preferably between two root
specific promoters. The DNA of the cloned gene under
the control of the root specific promoter can be
introduced in the plant of interest, using plant
transgenic technology. For every parasitic nematode, a
different piece of DNA may be required and likewise
for every plant race, a different promoter will be
needed.

The root will produce RNA or dsRNA from the
introduced piece of DNA when root specific promoter is
utilised. As the nematode feeds on the plant, the RNA
and/or dsRNA will be consumed or ingested by the
nematode. The RNA and/or dsRNA can enter the cells of
the nematode and perform its inhibitory action on the
target DNA. Depending on the nature of the cloned DNA
piece of worm, the nematode will not be able to
survive, to eat, proliferate, etc in any case
preventing the animal of feeding longer on the plant,
and thus protecting the plant.

- 30 -

Construction of a T7 RNA polymerase producing C.
elegans

5   To produce a T7 RNA polymerase or other RNA
polymerases in animals, and more particularly in
nematodes and most particularly in C. elegans, several
possibilities can be envisaged. The T7 RNA polymerase
can be expressed under various promoters. These
promoters may be inducible promoters, constitutive
10   promoters, general promoters, tissue specific
promoters, or combinations of those.

Example1:
Construction of a basic vector for expression of
15   T7 polymerase in C. elegans

The T7 polymerase coding sequence was PCR
amplified from_λ CE6 (Novagen, Madison, USA)using the
primers oGN26(ATGGAATTCTTACGCGAACGCGAAGTCCG) and
20   oGN46(CTCACCGGTAATGAACACGATTAACATCGC), using standard
procedures (PCR, A practical A practical approach,
1993, Ed. J. McPherson, et al, IRL Press). The
resulting DNA fragment encoding for the T7 RNA
polymerase was digested with AgeI and EcoRI and
25   inserted into the Fire vector pPD97.82 digested with
AgeI and EcoRI. The resulting construct encodes for an
open reading frame of T7 RNA polymerase in fusion with
the SV40 large T antigen nuclear localization signal
(NLS) with amino acid sequence MTAPKKKRKVPV. This
30   nuclear localization signal sequence is required to
translocate the T7 RNA polymerase from the cytoplasm
to the nucleus, where it is able to bind to its
specific promoters, designated T7 promoters. Upstream
of the coding sequence for the T7polymerasefusion
35   protein is a minimal promoter (myo-2) preceded by a

multiple cloning site (MCS) in which several C.
elegans promoters can be inserted. This plasmid
(pGN105 shown in Figure 11) is a basic T7 RNA
polymerase plasmid which enables the expression of
5   T7polymerase in C. elegans. Derivatives of this
plasmid wherein promoters are cloned into the multiple
cloning site, allow for the inducible, constitutive,
general and tissue specific expression of T7 RNA
polymerase in C. elegans, as expression will be
10   regulated by the promoter cloned in the multiple
cloning site.

Although not restricted to these examples, for
the following promoters it is known that they induce
expression in the following tissues.
15   let-858 (ubiquitous expression), myo-2 (pharynx
expression), myo-3 (body wall muscles), egl-15 (vulval
muscles), unc-119 (pan-neuron).

Example 2:
20   Construction of a vector for expression of T7 RNA
polymerase in C. elegans muscle tissue.

The T7 RNA polymerase coding sequence was PCR
amplified from_λ CE6 using the primers oGN43
25   (GCCACCGGTGCGAGCTCATGAACACGATTAACATCGC) and oGN44
(CACTAGTGGGCCCCTTACGCGAACGCGAAGTCCG) digested with
AgeI/SpeI and inserted in the pGK13 vector digested
with AgeI/SpeI. (This vector contains the strong SERCA
promoter which drives expression in the pharynx, the
30   vulval muscle, the tail and the body wall muscle). A
nuclear localization signal (NLS) of SV40 large T
antigen was inserted in front of the T7 polymerase
coding sequence by insertion of two overlapping
oligo's oGN45 (CCGGATGACTGCTCCAAAGAAGAAGCGTAAGCT) and
35   oGN46 (CTCACCGGTAATGAACACGATTAACATCGC) into the

- 32 -

SacI/AgeI restriction sites. The resulting construct
was called pGN108 as shown in Figure 10. Introduction
of this plasmid into C. elegans results in the
expression of T7 RNA polymerase in the pharynx, vulva
5   muscle, tail and body wall muscles.

To test expression and functionality of T7 RNA
polymerase in C. elegans under the regulation of the
SERCA promoter, pGN108, which encodes the T7RNA
polymerase under the control of the SERCA promoter was
10   injected into C. elegans. A test vector was co-
injected. This test vector encodes for GFP under the
control of a T7 promoter (pGN401 in Figure 13). The
plasmid pGN401 was constructed by inserting two
overlapping oligo's oGN41 (CCCGGGATTAATACGACTCACTATA)
15   and oGN42 (CCGGTATAGTGAGTCGTATTAATCCCGGGAGCT) in the
SacI/AgeI opened Fire vector pPD97.82. generating a T7
promoter. Furthermore a selection marker was co-
injected to select for transformants (rol6, pRF4). The
latter selection vector pRF4 is well known to person
20   skilled in the art. Transgenic F1 could easy be
isolated as they display the rol 6 phenotype. These
transgenic C. elegans all expressed GFP in the
pharynx, the vulval muscle, the tail and the body wall
muscle. This data show clearly that theT7 RNA
25   polymerase is functionally expressed under the
regulation of the SERCA promoter, and that the
expressed T7 RNA polymerase binds to the T7 promoter
present in pGN401 and initiates transcription of the
GFP gene, which is then functionally expressed,
30   resulting in fluorescence in the muscle tissues where
SERCA is inducing the expression of the T7 RNA
polymerase.

35

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Example 3:
Construction of a vector for ubiquitous
expression of T7 polymerase in C. elegans

5   The NLS-T7 RNA polymerase fusion gene was
isolated from pGN108 with XmaI/Bsp1201 and cloned into
the Fire vector pPD103.05 digested with
XmaI/Bsp120I.This results in a vector wherein the T7
RNA polymerase is cloned under the regulation of the
10   let858 promoter. This specific promoter enables the
expression of T7 RNA polymerase in all tissues. The
resulting plasmid was named **pGN110** (Figure 14).

Example 4:
15   Construction of a vector for T7 RNA polymerase
mediated expression of DNA fragments, genes, and
cDNA's under the control of a T7 promoter.

The Fire vector pPD97.82 was digested with SacI /
20   AgeI and a T7 promoter sequence was generated by
insertion of two overlapping oligo's oGN41
(CCCGGGATTAATACGACTCACTATA) and oGN42
(CCGGTATAGTGAGTCGTATTAATCCCGGGAGCT) into the SacI/Age/
restriction endonuclease sites. This construct (pGN400
25   Figure 12) contains a GFP open reading frame cloned
between SacI and EcoRI restriction endonuclease sites
under the regulation of the T7 promoter. Any gene,
cDNA, or DNA fragment can be cloned in this vector by
deleting the GFP gene as a AgeI/ SacI fragment and
30   cloning the DNA fragment of interest into the vector.
Preferentially the DNA fragment of interest can be
obtained by PCR amplification, inserting the SacI/AfeI
sites in the primers. The resulting DNA fragment after
PCR amplification is the digested and the GFP gene in
35   pGN400 is replaced by the amplified DNA fragment.

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Every vector that contains a T7 promoter could be used
for the purpose of T7 RNA polymerase induced
expression in C. elegans, such as the commercially
available pGEM vectors and the pBluescript vectors.
5   This is clearly shown by the pGN401 vector which
expresses GFP under the regulation of the T7 promoter
in a transgenic C. elegans which expresses T7 RNA
polymerase.
        The use of pGN400 has the advantage that the
10   vector includes a 3'UTR fragment from unc-54 which
enhances the transcription or stability of the RNA.

        **Generation of permanent, tissue specific "pseudo
        knock-out" RNAi C. elegans lines**
15

        At present, gene knock outs in C. elegans are
obtained after random, large scale mutagenesis and PCR
base sib-selection. This method is bulky, very time
consuming and tedious. It has been described that
20   introducing double stranded RNA into a cell results in
potent and specific interference of expression of
endogenous genes. In C. elegans gene expression can be
down regulated by injection of RNA into the body
cavity of the worm, soaking the worm in a solution
25   containing dsRNA or feeding E. coli that express dsRNA
corresponding to the gene of interest. C. elegans
cells have the ability to take in dsRNA from their
extracellular environment. It has been reported that
mRNA is the target of this ds RNA mediated genetic
30   interference (Montgomery and Fire 1998). It is also
suggested that the targeted RNA is degraded in the
nucleus before translation can occur. Although the
RNAi mediated reduction of gene expression can be
passed on to the next generations, heritability is
35   poor and the effect is rapidly lost during further

- 35 -

offspring. This is probably due to a continued

decrease of the dsRNA pool. We propose here a method

to construct C. elegans lines with a permanent,

inheritable, RNAi phenotype. The method encompasses

5   the generation of transgenic C. elegans lines by

introducing plasmids containing cDNA fragments of the

target gene in the sense and antisense orientation

under control of a worm promoter or by transcription

of an inverted repeat of the cDNA from a single

10 construct. Alternatively, ds RNA can be transcribed

from a vector harboring a cDNA flanked by two T7

promoters in a C. elegans strain that expresses T7

polymerase. The result is a transgenic worm with an

heritable stable "pseudo knock-out" phenotype. The

15 expression of the cDNA or the T7 polymerase can be

general and constitutive but could also be regulated

under a tissue specific promoter. In contrast to RNAi

induced by external ds RNAi (injected, soaked or

feeded) this method would enable to obtain

20 conditional, tissue specific inhibition of gene

expression.

Inhibition of unc-22 expression by RNA

interference results in a "twitching" phenotype.

25

Unc 22 cDNA (exon 22) was cloned in sense and

antisense orientation in pPD103.05. (A. Fire nr L2865)

containing the let 858 promoter that is capable of

expressing RNA sequences in all tissues. The resulting

30 plasmids were named pGN205 (Figure 19a) and pGN207

(Figure 19 b). These constructs were introduced into

C. elegans together with a selectable marker (rol-6;

GFP). Transgenic F1 individuals (expressing rol-6 or

GFP) showed a "twitching" phenotype indicating that

35 RNAi could be mediated by endogenous transcription of

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RNA from transgenic DNA. The RNAi phenotype co-
segregated with the selectable marker into further
offspring. This resulted in the generation of C.
elegans lines with permanent RNAi phenotype.

5

Generation of stable lines T7 RNA polymerase
lines and generation of dual transgenic worms.

An expression system in C. elegans based on an
10   exogenous RNA polymerase demands two plasmids. One is
encoded for the RNA polymerase under the control of a
specific promoter, while the other plasmid encodes for
the DNA fragment to be expressed, under the regulation
of the T7 promoter. In the case of semi stable RNAi
15   also designated pseudo stable knockouts, the DNA of
interest is cloned between two T7 promoters so that
dsRNA can be produced.
As the T7 RNA polymerase expression system is
known to be a high expression system this will result
20   in problems to generate dually transgenic animals. If
the gene to be expressed in the C. elegans nematode is
toxic, this will result in lethal effects and hence in
the construction of a C. elegans without highly
regulated stable expression of the gene of interest.
25   If the gene of interest is essential for the survival
of the organism, RNAi with a DNA fragment from this
gene will also result in lethal effects, so that
pseudo- stable knockouts are not possible.
To overcome this problem the present inventors
30   have designed a system consisting of two transgenic
animals. The first animal is transgenic for the T7 RNA
polymerase, This T7 RNA polymerase can be expressed in
all cells or specific cells or tissues as has been
shown in previous examples. The second transgenic
35   animal is transgenic for the DNA fragment of interest.

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This can be a gene or cDNA linked to a T7 promoter, or if one wants to perform RNAi a DNA fragment of such gene cloned between two T7 promoters.

Both transgenic animals are viable and do not show any aberrant phenotypes. This is because the T7RNA polymerase expressed in the first transgenic organism is not toxic for the organism, even if expressed at relative high levels. In the second transgenic organism, the gene of interest is not expressed or the dsRNA is not produced as these transgenic animals do not contain the T7 RNA polymerase.

Expression of the gene or cDNA of interest or RNAi with a DNA fragment can now be obtained by mating the two transgenic animals. The offspring of these are dually transgenic and express the gene of interest or express dsRNa of the DNA fragment of interest.
To generate sufficient males in such a mating, one of the transgenic animals males can be a C. elegans mutant with a phenotype favouring generation of males. An Example of such a mutant is him-5. Preferentially such a mutant will be used to make a C. elegans transgenic for T7 RNA polymerase, while the hermaphrodite harbors the DNA fragment under the regulation of the T7 promoter.

To select efficiently for the dual transgenic offspring a second transgene can be introduced in the second transgenic animal. This transgene contains a reporter gene under the regulation of the T7 promoter. The reporter gene can be GFP, luciferase, Beta galmactosidase, or beta-lactamase. an example of such a ttransgene are the vectors pGN400 and pGN401.

To obtain inducible, tissue specific expression of a transgene in C. elegans we can make male stock (i.e. him-5) carrying the T7 polymerase construct

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under the control of different C. elegans promoters
that enable tissue specific expression such as). This
males can be crossed with hermaphrodites carrying the
gene of interest under the control of a T7 promoter.
5   Furthermore, the transgenes can be integrated
into the genome of the animal. Methods to generate
stable integration of a plasmid into the genome of the
animal have been described (Methods in cell biology,
Vol. 48, 1995, ed. by epstein and Shakes, academic
10   press) and involve radiation of the animal.
This can be done for both animals animals, but
preferentially, the animals expressing the T7 RNA
polymerase are subject to such traetment. This result
in a collection of C. elegans nematodes that stably
15   express T7 RNA polymerase under the control of various
.promoters. examples of such promoters are the myo-2
(pharynx expression), myo-3 (body wall muscles), egl-
15 (vulval muscles), unc-119 (pan-neuron), SERCA
(muscles), let858 (all cells) ges-1 (gut).
20

        Construction of RNAi T7 promoter yeast two hybrid
        vectors

        pGAD424 with forward and reverse T7/T3 and or Sp6
25   In most two-hybrid experiments a cDNA library is
cloned in plasmid pGAD424 (Figure 16) which has been
engineered with additional restriction sites in the
polylinker such as a NcoI site (Clontech). This
library allows for screening of binding proteins in a
30   yeast two hybrid experiment. We constructed a new
yeast two hybrid vector with the same possiblilities
to perform yeast two hybrid, but which contain two
addditional T7 promoters, so that the vector can be
used for T7 RNA polymerase induced pseudo-stable
35   knock-outs. For this we inserted a forward T7 by using

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a T7- linker (consisting of the following primers
aattcttaatacgactcactatagggcc and
catgggccctatagtgagtcgtattaag) into the EcoRI-Ncol site
of pGAD424. The resulting vector was designated
pGAD424-without-FULL-ICE-both-T7. Care was taken to
eliminate stop codons and using maximal polylinker
compatible amino acids. We adopted the same strategy
for the reverse T7 (consisting of both primers
gatccgtcgacagatctccctatagtgagtcgtattactgca and
gtaatacgactcactatagggagatctgtcgacg) with BamH1 and
Pst1. To avoid loss of SalI, we included this site in
the primer.

The SalI site is important as most libraries are
cloned in this site, adapters are available. This
makes the newly constructed vector compatible with
existing vectors.

   pAS2 with with forward and reverse T7/T3 and or
   Sp6

   An analogous yeast two hybrid vector was
constructed based on pAS2 (Clontech). By partial EcoRV
digestion we were able to remove a significant part of
the cyh2 gene. The right construct can be isolated and
checked by a restriction digest with BglII. this
restriction site is present in the EcoRV fragment of
PAS2 to be eliminated. This elimates the cyh2 gene
which is slightly toxic gene and involved in growth
retardation. This gene is non-essential for the
performing of RNAi and Yeast two hybrid experiments.
After the elimination of the EcoRV fragment, The
EcoRI restriction site which is located between the
DNA sequence encoding for GAL4DB and HA (epitope)
becomes unique for the plasmid, and can be used to
subsitute HA with a T7 promoter containing linker.

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This ensures persistence of all restriction sites,
allowing both in frame cloning and compatibility with
previous vectors and pGAD424. We used the following
linker (primers: aattcttaatacgactcactatagggca and

5   tatgccctatagtgagtcgtattaag) using EcorI and NdeI
cloning sites. We adopted the same strategy for the
reverse T7 (primers:
gatccgtcgacagatctccctatagtgagtcgtattactgca
catgggccctatagtgagtcgtattaag and

10   gtaatacgactcactatagggagatctgtcgacg) with BamH1 and
Pst1. To avoid loss of Sal1 we included it in the
primer.The resulting vector was designated pAS2-cyh2-
HA+both T7-final.

       Having the T7 promoter (or alternatively the T3,

15   or SP6 promoter) in pGAD424 allows to go quickly from
interacting protein to RNAi and assigning function to
the isolated DNA fragment. An additional advantage is
the ability to make by in vitro transcription coupled
to in vitro translation (There is an ATG in frame with

20   either GAL4DB or GAL4AD) labeled protein which can be
used for in vitro controls (e.g. pull down assays) of
the actual protein-protein interaction.

       The sequences of the plasmids produced and the
SP6 and T3 polymerase are identified in the Sequence

25   Listing provided below:

SP6 DNA-dependent RNA polymerase: SEQUENCE ID NO. 1

swissprot accesion number P06221

protein sequence:

```
  1 mqdlhaiqlq leeemfnggi rrfeadqqrq iaagsesdta wnrrllseli apmaegiqay
 61 keeyegkkgr apralaflqc venevaayit mkvvmdmlnt datlqaiams vaeriedqvr
121 fskleghaak yfekvkkslk asrtksyrha hnvavvaeks vaekdadfdr weawpketql
181 qigttlleil egsvfyngep vfmramrtyg gktiyylqts esvgqwisaf kehvaqlspa
241 yapcvipprp wrtpfnggfh tekvasrirl vkgnrehvrk ltqkqmpkvy kainalqntq
301 wqinkdvlav ieevirldlg ygvpsfkpli dkenkpanpv pvefqhlrgr elkemlspeq
361 wqqfinwkge carlytaetk rgsksaavvr mvgqarkysa fesiyfvyam dsrsrvyvqs
421 stlspqsndl gkallrfteg rpvngvealk wfcinganlw gwdkktfdvr vsnvldeefq
481 dmcrdiaadp ltftqwakad apyeflawcf eyaqyldlvd egradefrth lpvhqdgscs
541 giqhysamlr devgakavnl kpsdapqdiy gavaqvvikk nalymdadda ttftsgsvtl
601 sgtelramas awdsigitrs ltkkpvmtlp ygstrltcre svidyivdle ekeaqkavae
661 grtankvhpf eddrqdyltp gaaynymtal iwpsisevvk apivamkmir qlarfaakrn
721 eglmytlptg fileqkimat emlrvrtclm gdikmslqve tdivdeaamm gaaapnfvhg
781 hdashliltv celvdkgvts iavihdsfgt hadntltlrv alkgqmvamy idgnalqkll
841 eehevrwmvd tgievpeqge fdlneimdse yvfa
```

T3 DNA dependent RNA polymerase: SEQUENCE ID NO. 2

swissprot accession number P07659

protein sequence:

```
  1 mniieniekn dfseielaai pfntladhyg salakeqlal ehesyelger rflkmlerqa
 61 kageiadnaa akpllatllp klttrivewl eeyaskkgrk psayaplqll kpeasafitl
121 kvilasltst nmttiqaaag mlgkaiedea rfgrirdlea khfkkhveeq lnkrhgqvyk
181 kafmqvvead migrgllgge awsswdkett mhvgirliem liestglvel qrhnagnags
241 dhealqlaqe yvdvlakrag alagispmfq pcvvppkpwv aitgggywan grrplalvrt
301 hskkglmrye dvympevyka vnlaqntawk inkkvlavvn eivnwkncpv adipslerqe
361 lppkpddidt neaalkewkk aaagiyrldk arvsrrisle fmleqankfa skkaiwfpyn
421 mdwrgrvyav pmfnpqgndm tkglltlakg kpigeegfyw lkihgancag vdkvpfperi
481 afiekhvddi lacakdpinn twwaeqdspf cflafcfeya gvthhglsyn cslplafdgs
541 csgiqhfsam lrdevggrav nllpsetvqd iygivaqkvn eilkqdaing tpnemitvtd
601 kdtgeisekl klgtstlaqq wlaygvtrsv tkrsvmtlay gskefgfrqq vlddtiqpai
661 dsgkglmftq pnqaagymak liwdavsvtv vaaveamnwl ksaakllaae vkdkktkeil
721 rhrcavhwtt pdgfpvwqey rkplqkrldm iflgqfrlqp tintlkdsgi dahkqesgia
781 pnfvhsqdgs hlrmtvvyah ekygiesfal ihdsfgtipa dagklfkavr etmvityenn
841 dvladfysqf adqlhetqld kmpplpkkgn lnlqdilksd fafa
```

pGN108:   SEQUENCE ID NO. 3

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pGN105:   SEQUENCE ID NO. 4

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agggaacaaaagctggggggggatcctccaaaatcgtcttccgctctgaaaaacgaaagtggaccttttgacatccgaaaaaatgggcgaaaaatga
aattgagcttttttgggtcgaaaaaaatgtttttagaatgctgagaacacgttaaacacgaagatcatatttattttgagacccggatgctctgaaaatgtctg
acatagatttaaaaaaagcafatatatatttttcattttcaacgtgaaagttttgtgcaacttatagaatctcctattggcacattgttttttatttaactgaggcag
ttttttgaacacctttttgaaactttgaatctctttgaagtatactgtcgaaaagactgacttgagcgttcgaaatgccagaagaaaactatatttgaatctcgc
gctaaattgagaaatgcaaccgcgctccactggacaattggaaaaaaaaattattcggaggcgacaacggtatttttcgaaattgattttctgtgtatttct
cattttttataaattcttctttgatttatcgttcgtttgtgagaaatttaattgtattcaaactttttatagtaagata

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Claims

1.   A method of identifying DNA responsible for
conferring a particular phenotype in a cell which
5   method comprises
a)   constructing a cDNA or genomic library of
the DNA of said cell in a suitable vector in an
orientation relative to a promoter(s) capable of
initiating transcription of said cDNA or DNA to double
10   stranded (ds) RNA upon binding of an appropriate
transcription factor to said promoter(s),
b)   introducing said library into one or more of
said cells comprising said transcription factor, and
c)   identifying and isolating a particular
15   phenotype of said cell comprising said library and
identifying the DNA or cDNA fragment from said library
responsible for conferring said phenotype.

2.   A method according to claim 1 wherein said
20   library is organised into hierarchical pools prior to
step b).

3.   A method of assigning function to a known
DNA sequence which method comprises
25   a)   identifying a homologue(s) of said DNA
sequence in a cell,
b)   isolating the relevant DNA homologue(s) or a
fragment thereof from said cell,
c)   cloning said homologue or fragment thereof
30   into an appropriate vector in an orientation relative
to a suitable promoter(s) capable of initiating
transcription of dsRNA from said DNA homologue or
fragment upon binding of an appropriate transcription
factor to said promoter(s),
35   d)   introducing said vector into said cell from

step a) comprising said transcription factor, and

e)   identifying the phenotype of said cell
compared to wild type.

5   4.   A method according to any of claims 1 to 3
wherein said DNA library, homologue or fragment is
cloned in a sense and an antisense direction relative
to said promoter.

10   5.   A method according to any of claims 1 to 3
wherein said DNA library, homologue or fragment is
cloned between two promoters capable of producing
dsRNA from said DNA library, homologue or fragment
upon binding of said transcription factor to said
15   promoters.

6.   A method according to any of claims 1 to 5
wherein said cell is adapted to express said
transcription factor.

20

7.   A method according to any of claims 1 to 6
wherein said DNA library, homologue or fragment is
constructed in a suitable vector which comprises a
sequence of nucleotides encoding said transcription
25   factor operably linked to a suitable promoter.

8.   A method according to any of claims 1 to 6
wherein said transcription factor is encoded by a
further vector independent of the vector including
30   said DNA library, DNA homologue or fragment and which
sequence encoding said transcription factor is
operably linked to a suitable promoter.

9.   A method according to claim 7 or 8
35   wherein said transcription factor comprises any of T7,

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T3 or SP6 polymerase.

10.   A method according to claim 7 or 8 wherein
said suitable promoter comprises any of let 858,
SERCA, UL6, myo-2 or myo-3.

11.   A method according to any of claims 7 to 10,
wherein said suitable vector or said further vector
comprises a selectable marker.

12.   A method according to claim 11 wherein said
selectable marker comprises a nucleotide sequence
capable of inhibiting or preventing expression of a
gene in said cell and which gene is responsible for
conferring a known phenotype.

13.   A method according to claim 12 wherein said
nucleotide sequence comprises a sequence which is a
part of or identical to said gene conferring said
phenotype, and which nucleotide sequence is itself
oriented relative to a suitable promoter(s) capable of
initiating transcription of double stranded RNA upon
binding of an appropriate transcription factor to said
promoters.

14.   A method according to claim 12 wherein said
nucleotide sequence is a part of or identical to said
gene sequence conferring said phenotype, and which
nucleotide sequence is such as to permit integration
of said suitable or further vector by homologous
recombination in the genome of said cell and following
said integration said nucleotide sequence is capable
of inhibiting expression of said gene sequence
conferring said phenotype.

15.   A method according to claim 14 wherein said
nucleotide sequence comprises stop codons sufficient
to prevent translation of said nucleotide sequence
following its integration into said genome.

5

16.   A method according to any preceding claim
wherein said cell is a microorganism suitable for
feeding to, transforming or infecting an organism.

10   17.   A method according to any of claims 1 to 14
wherein said cell is contained in an organism or an
embryo thereof.

18.   A method according to any of claims 1 to 17
15   wherein said promoters are T7 promoters.

19.   A method according to any of claims 12 to 18
wherein said known gene sequence comprises a sup-35
gene or a fragment thereof which is selectable by
20   identifying offspring growing at a temperature above
25°C following introduction of said vector in the
genome of a pha-1 et123ts mutant *C. elegans* worm.

20.   A method according to any of claims 1 to 19
25   wherein said cell or organism is contacted with a
specified compound for screening for a desired
phenotype, such as resistance or sensitivity to said
compound when compared to the wild type cell or
organism.

30

21.   A method according to any preceding claim
wherein said transcription factor is inducible.

22.   A method according to claim 16 wherein said
35   microorganism is an *E. coli* strain which is an RNAase

III and preferably an RNAase negative strain.

23. A method according to any of claims 17
wherein said organism is selected from the class
5   nematoda.

24. A method according to claim 23 wherein said
organism is *Caenorhabditis elegans*.

10   25. A method of generating a transgenic non-
human organism comprising an exogenous transcription
factor and a transgene comprising a promoter operably
linked to DNA fragment which is expressed upon binding
of said transcription factor thereto, the method
15   comprising
         a)   providing a first transgenic organism
comprising a first construct incorporating DNA
encoding an exogenous transcription factor and a
second transgenic organism comprising a second
20   construct including at least one promoter operably
linked to a desired DNA sequence which is expressed
upon binding of the transcription factor of said first
transgenic organism thereto,
         b)   crossing said first and second transgenic
25   organisms and selecting offspring expressing said
desired DNA sequence.

26. A method according to claim 25 wherein said
first and second transgenic organisms are generated by
30   transforming said first and second constructs into
respective microorganisms for subsequent feeding to
the respective organism.

27. A method according to claim 25 or 26 wherein
35   said second construct comprises said desired DNA

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sequence in an orientation relative to said promoter
so as to be capable of initiating transcription of
said DNA to dsRNA upon binding of said transcription
factor thereto.

5

28.   A method according to claim 27 wherein said
second construct comprises two promoters flanking said
desired DNA sequence which promoters can initiate
transcription of said DNA sequence to dsRNA upon
10   binding of said transcription factor to said
promoters.

29.   A method according to claim 27 wherein said
DNA sequence is provided in a sense and an antisense
15   orientation relative to said promoter so as to produce
dsRNA upon binding of the transcription factor to the
promoter.

30.   A method according to any of claims 25 to 29
20   wherein said second transgenic organism further
comprises a reporter gene operably linked to a
promoter which is capable of initiating transcription
of said reporter upon binding of said transcription
factor thereto.

25

31.   A method according to any of claims 25 to 30
wherein said transcription factor comprises a
polymerase.

30   32.   A method according to claim 31 wherein said
polymerase comprises any of T7, T3 or SP6 polymerase.

33.   A method according to any of claims 25 to 31
wherein said promoters comprises any of T7, T3 or SP6
35   promoters.

34.   A method according to claim 30 wherein said
reporter gene comprises any of those sequence encoding
Luciferase, Green Fluorescent protein, β galactosidase
or β-lactamase.

5

35.   A method according to any of claims 25 to 30
wherein said organism is of the species nematoda.

36.   A method according to claim 35 wherein said
10   nematoda species is *C. elegans*.

37.   A transgenic non-human multicellular
organism obtainable according to the methods of any
one of claims 25 to 34.

15

38.   A method of validating clones identified in
yeast two hybrid vector experiments which method
comprises
       a)   providing a construct including the DNA
20   encoding the protein identified in the two hybrid
vector experiment, which construct is such that said
DNA is orientated relative to a promoter(s) that is
capable of initiating transcription of said DNA to
double stranded RNA upon binding of an appropriate
25   transcription factor to said promoter(s),
       b)   transforming a cell comprising said
transcription factor with said construct, and
       c)   identifying a phenotypic change in said cell
or organism when compared to a wild type.

30

39.   A method according to claim 38 wherein said
                     DNA sequence is provided between two
                     promoters capable of initiating
                     transcription of the DNA sequence to dsRNA
35   upon binding of the transcription factor to

said promoters.

40. A method according to claim 38 wherein said
DNA is provided in a sense and an antisense
orientation relative to said promoter such that
binding of the transcription factor to said promoter
initiates transcription of dsRNA from said DNA.

41. A method according to any of claims 38 to 40
wherein said transcription factor is inducible in said
cell.

42. A method according to any of claims 38 to 41
wherein said promoter is a phage polymerase promoter
and said transcription factor is a RNA polymerase.

43. A method according to claim 42 wherein said
polymerase is any of T7 RNA polymerase, T3 RNA
polymerase or SP6 RNA polymerase.

44. A method according to claim 43 wherein said
promoters comprise any of T7, T3 or SP6 promoter

45. A method according to any of claims 38 to 44
wherein said construct is such that it may be used in
yeast two hybrid experiments.

46. A method according to any of claims 38 to 45
wherein said cell is an *E. coli* cell.

47. A method according to any of claims 38 to 45
wherein said cell is part of an organism or an embryo
thereof.

48. A method according to claim 47 wherein said

organism is of the species nematoda and preferably *C. elegans.*

49.   Plasmid pGN1 as illustrated in Figure 1.

50.   Plasmid pGN100 as illustrated in Figure 2.

51.   The yeast two hybrid vector plasmid illustrated in any of Figures 4, 15 or 16(Seq ID Nos 8 and 9).

52.   A plasmid as illustrated in Figure 7.

53.   A plasmid as illustrated in Figure 8.

54.   A method of alleviating infestation of plant pests, which method comprises

a)   identifying a DNA sequence from said pest which is critical for its survival, growth, proliferation,

b)   cloning said sequence from step a) or a fragment thereof in a suitable vector in an orientation relative to promoter(s) such that said promoter(s) is capable of initiating transcription of said DNA sequence to RNA or dsRNA upon binding of an appropriate transcription factor to said promoter(s), and

c)   introducing said vector into the plant.

55.   A method according to claim 54 wherein said DNA sequence is provided between two promoters such that binding of the transcription factor to the promoters results in transcription of the DNA to dsRNA.

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56. A method according to claim 54 wherein said DNA sequence is provided in a sense and an antisense orientation relative to said promoter such that binding of the transcription factor to the promoter
5   results in transcription of the DNA to dsRNA.

57. A method according to claim 54 wherein said pest is a nematode worm.

10   58. A method according to claim 57 wherein said nematode comprises any of Tylenchulus ssp. Radopholus ssp., Rhadinaphelenchus ssp., Heterodera ssp., Rotylenchulus ssp., Pratylenchus ssp., Belonolaimus ssp., Canjanus ssp., Meloidogyne ssp., Globodera ssp.,
15   Nacobbus ssp., Ditylenchus ssp., Aphelenchoides ssp., Hirschmenniella ssp., Anguina ssp., Hoplolaimus ssp., Heliotylenchus ssp., Criconemellassp., Xiphinemassp., Longidorus ssp., Trichodorus ssp., Paratrichodorus ssp., Aphelenchs ssp.
20

59. A method according to claim 55 wherein said DNA sequence or fragment thereof is cloned between two tissue, preferably root specific promoters.

25   60. An expression vector for use in a method according to any proceeding claim comprising a promoter or promoters oriented relative to a DNA sequence such that they are capable of initiating transcription of said DNA sequence to double stranded
30   RNA upon binding of an appropriate transcription factor to said promoter or promoters.

61. An expression vector according to claim 60 comprising two identical promoters flanking the DNA
35   sequence.

62. An expression vector according to claim 60
comprising said DNA sequence in a sense and an
antisense orientation relative to said promoter.

5   63. An expression vector according to any of
claims 60 to 62 which further comprises a nucleotide
sequence encoding a selectable marker.

64. An expression vector according to claim 63
10   wherein said nucleotide sequence encoding said
selectable marker is orientated relative to the
promoter(s) such that transcription of the nucleotide
sequence to double stranded RNA occurs upon binding of
an appropriate transcription factor to said
15   promoter(s).

65. An expression vector according to claim 64
wherein said nucleotide sequence encoding the
selectable marker is provided between the identical
20   promoters capable of initiating transcription of the
nucleotide sequence to dsRNA upon binding of the
transcription factor to the promoters.

66. An expression vector according to claim 64
25   wherein said nucleotide sequence encoding the
selectable marker is provided in a sense and an
antisense orientation relative to the promoter such
that transcription of the nucleotide sequence to dsRNA
upon binding of the transcription factor to said
30   promoter occurs.

67. An expression vector according to claim 63
or 64 wherein said selectable marker comprises a
nucleotide sequence encoding sup-35, for introduction
35   into *C. elegans* having a pha-1 mutation.

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68. An expression vector for expressing an appropriate transcription factor for use in a method according to any of claims 1 to 48 and 54 to 59 which vector comprises a sequence of nucleotides encoding said transcription factor operably linked to suitable expression control sequences.

69. An expression vector according to claim 68 wherein said expression control sequences include promoters which are inducible, constitutive, general or tissue specific promoters, or combinations thereof.

70. An expression vector according to any of claims 68 to 69 wherein said transcription factor comprises a phage polymerase, and preferably T7 RNA polymerase.

71. An organism or cell transformed or transfected with a plasmid according to any of claims 49 to 53 or an expression vector according to any of claims 60 to 70.

72. An organism according to claim 71, which is of the species nematoda and preferentially *C. elegans*.

73. A method of introducing dsRNA or DNA capable of producing dsRNA into an organism which method comprises feeding said organism with a suitable microorganism comprising an expression vector according to any of claims 60 to 67 or feeding said organism directly with an expression vector according to any of claims 60 to 67.

74. A method according to claim 73 wherein said microorganism or said organism is adapted to express

said transcription factor.

75.   A method according to claim 74 wherein
either said microorganism or said organism comprises
an expressing vector according to any of claims 66 to
69.

76.   A method according to any of claims 73 to 75
wherein said organism is *C. elegans* and said
microorganism is *E. coli*.

77.   A method according to claim 76 wherein said
E. coli strain is an RNAaseIII negative strain.

78.   A method according to any of claims 73 to 75
wherein said organism is a *C. elegans* nuc-1 mutant.

79.   A selection system for identifying
transformation of a cell or organism with a vector
according to claims 60 to 63 which system comprises a
vector according to claims 60 to 63 and said
selectable marker comprises a nucleotide sequence
capable of inhibiting or preventing expression of a
gene in said cell or organism which gene is
responsible for conferring a known phenotype.

80.   A selection system according to claim 79
wherein said nucleotide sequence comprises a sequence
which is a part of or identical to said gene
conferring said known phenotype, and which nucleotide
sequence is itself located between two identical
promoters capable of initiating transcription of
double stranded RNA upon binding of an appropriate
transcription factor to said promoters.

81. A selection system according to claim 79
wherein said nucleotide sequence is a part of or
identical to said gene sequence which confers a known
phenotype on said cell or organism, and which
5   nucleotide sequence permits integration of said vector
by homologous recombination in the chromosome of said
cell or organism and following said integration said
sequence inhibits expression of said gene sequence
conferring said known phenotype.
10

82. A selection system according to claim 81
wherein said nucleotide sequence comprises stop codons
sufficient to prevent translation of said nucleotide
sequence following integration into said genome.
15

83. A selection system according to claim 79
wherein said known gene sequence comprises a sup-35
gene or a fragment thereof which is selectable by
identifying offspring growing at a temperature above
20   25°C following introduction of said vector in a pha-1
et123ts mutant C. elegans worm.

84. A method according to claim 74 wherein said
transcription factor is T7 RNA polymerase.
25

85. A method of assigning function to a DNA
sequence of a multicellular organism which method
comprises:
a) providing:
30   (i)   a first construct comprising said DNA
sequence cloned in a sense direction under the
regulation of a suitable promoter
(ii)   a second construct comprising said DNA
sequence cloned in an anti-sense direction under the
35   regulation of the same promoter as in step (i),

in a multicellular organism which is adapted
to initiate transcription of said DNA fragment from
said promoter,

b)   identifying the phenotype of said
multicellular organism compared to wild type.

86.   A method of assigning function to a DNA
sequence of a multicellular organism which method
comprises:

a)   providing

i)   a construct comprising said DNA fragment
cloned between two promoters capable of promoting
transcription in said multicellular organism,

in a multicellular organism capable of
initiating transcription from said promoter;

b)   identifying the phenotype of said
multicellular organism compared to wild type.

87.   A method for expressing a gene, cDNA or
DNA fragment in C. elegans which method comprises :

i) providing a transgenic C. elegans
expressing an exogenous transcription factor,

ii) cloning said gene or cDNA or DNA fragment
into an appropriate vector operably linked to a
promoter capable of initiating transcription of said
gene, cDNA or DNA fragment upon binding of said
exogenous transcription factor thereto,

iii) introducing said vector into said
transgenic C. elegans from step i).

88.   A method according to claim 87 wherein
said exogenous transcription factor is a phage
polymerase.

89.   A method according to claim 88 wherein

said phage polymerase is T3, SP6 or T7 RNA polymerase.

     90.   A method according to any of claims 87 to 89 wherein said promoter is any of T3, T7 or SP6 promoter.

FIG. 1.

pGN1
gagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggcgaaattgtaaacgttaatattt
tgttaaaaattcgcgttaaatatttgttaaatcagctcattttttaaccaataggccgaaatcggcaaaatcccttataaatcaaaagaat
agaccgagatagggttgagtgttgttccagtttggaacaagagtccactattaaagaacgtggactccaacgtcaaagggcgaa
aaaccgtctatcagggcgatggcccactacgtgaaccatcacccaaatcaagtttttgcggtcgaggtgccgtaaagctctaaat
cggaaccctaaaggagcccccgatttagagcttgacggggaaagccggcgaacgtggcgagaaaggaagggaagaaag
cgaaaggagcgggcgctagggcgctggcaagtgtagcggtcacgctgcgcgtaaccaccacacccgccgcgcttaatgcgc
cgctacagggcgcgtccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgctattacgcca
gctggcgaaaggggggatgtgctgcaaggcgattaagttgggtaacgccaggtttttcccagtcacgacgttgtaaaacgacgg
ccagtgaattgtaatacgactcactatagggcgaattcgagctcggtacccggggatcctctagagtcgaaagcttctcgccctat
agtgagtcgtattacagcttgagtattctatagtgtcacctaaatagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgtt
atccgctcacaattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacatt
aattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggag
aggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcag
ctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaa
ggccaggaaccgtaaaaaggccgcgttgctggcgttttttcgataggctccgcccccctgacgagcatcacaaaaatcgacgct
caagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccctcgtgcgctctcctgttc
cgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctca
gttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaacta
tcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgt
aggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagc
cagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttttttgtttgcaagcagc
agattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgt
taagggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatat
atgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcct
gactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgct

FIG. 1. (CONTINUED)

caccggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctcc
atccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttggcattgctacaggca
tcgtggtgtcacgctcgtcgttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtg
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gcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaataccgcgccc
ggcgaccgagttgctcttgcccggcgtcaatacgggataatagtgtatgacatagcagaactttaaaagtgctcatcattggaaaa
cgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttca
gcatctttactttcaccagcgttctggggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacg
gaaatgttgaatactcatactcttccttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtat
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aacctataaaaataggcgtatcacgaggcccttcgtctcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcc
cggagacggtcacagcttgtctgtaagcggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggt
gtcggggctggcttaactatgcggcatcagagcagattgtactga

FIG. 2.

PGN100

ctagcatgaacacgattaacatcgctaagaacgacttctctgacatcgaactggctgctatcccgttcaacactctggctgaccatt
acggtgagcgtttagctcgcgaacagttggcccttgagcatgagtcttacgagatgggtgaagcacgcttccgcaagatgtttga
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caactcaacaagcgcgtagggcacgtctacaagaaagcatttatgcaagttgtcgaggctgacatgctctctaagggtctactcg
gtggcgaggcgtggtcttcgtggcataaggaagactctattcatgtaggagtacgctgcatcgagatgctcattgagtcaaccgg
aatggttagcttacaccgccaaaatgctggcgtagtaggtcaagactctgagactatcgaactcgcacctgaatacgctgaggct
atcgcaacccgtgcaggtgcgctggctggcatctctccgatgttccaaccttgcgtagttcctcctaagccgtggactggcattac
tggtggtggctattgggctaacggtcgtcgtcctctggcgctggtgcgtactcacagtaagaaagcactgatgcgctacgaagac
gtttacatgcctgaggtgtacaaagcgattaacattgcgcaaaacaccgcatggaaaatcaacaagaaagtcctagcggtcgcc
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gcggtcgtgtttacgccgtgtcaatgttcaacccgcaaggtaacgatatgaccaaaggactgcttacgctggcgaaaggtaaac
caatcggtaaggaaggttactactggctgaaaatccacggtgcaaactgtgcgggtgtcgataaggttccgttccctgagcgcat
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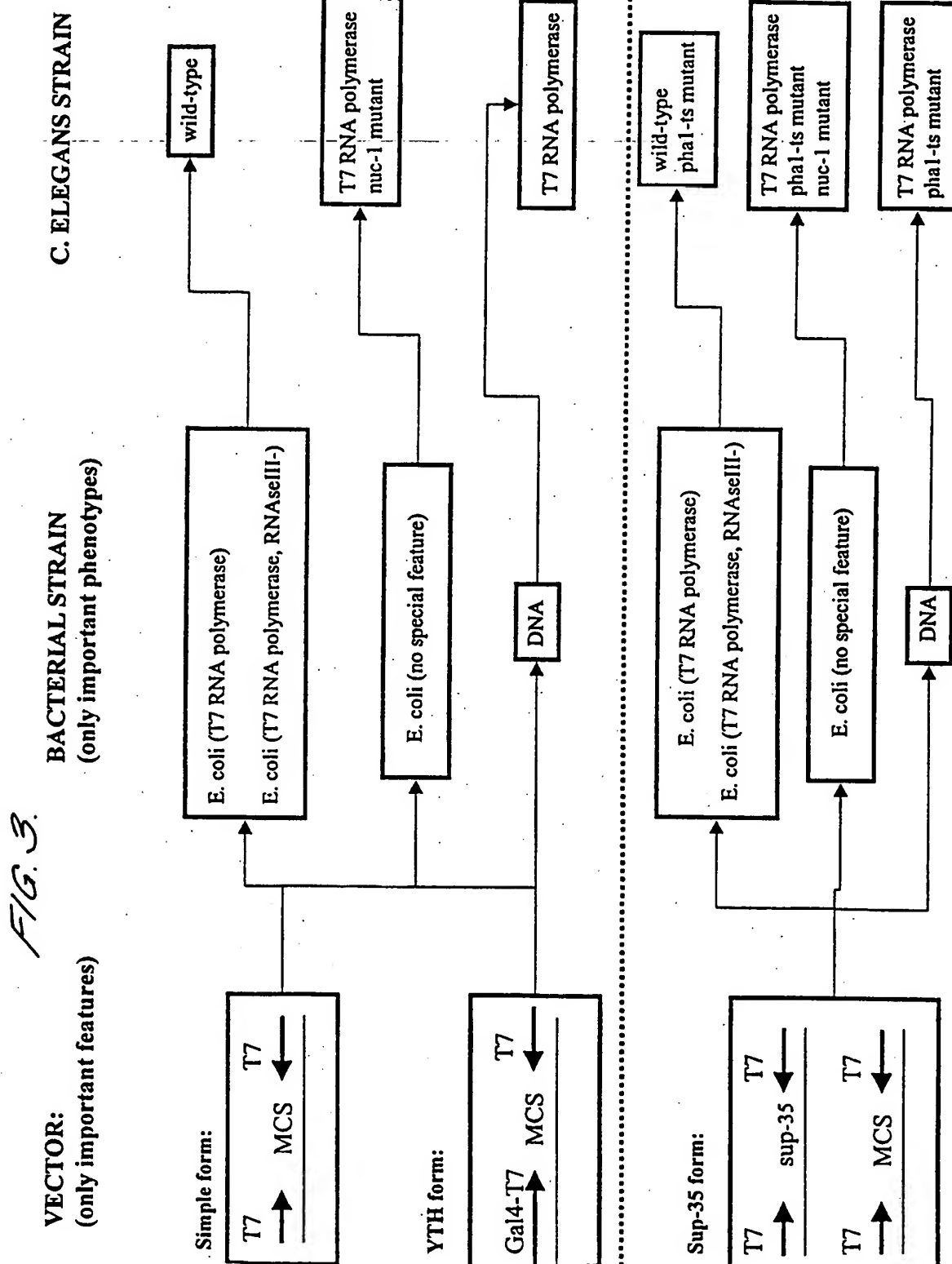
3/21

FIG. 2. (CONTINUED 1)

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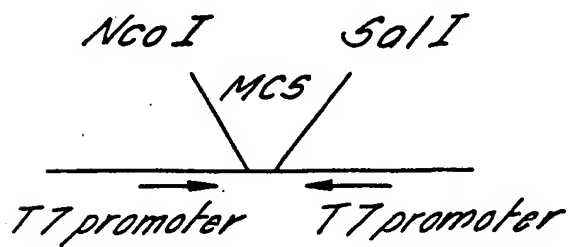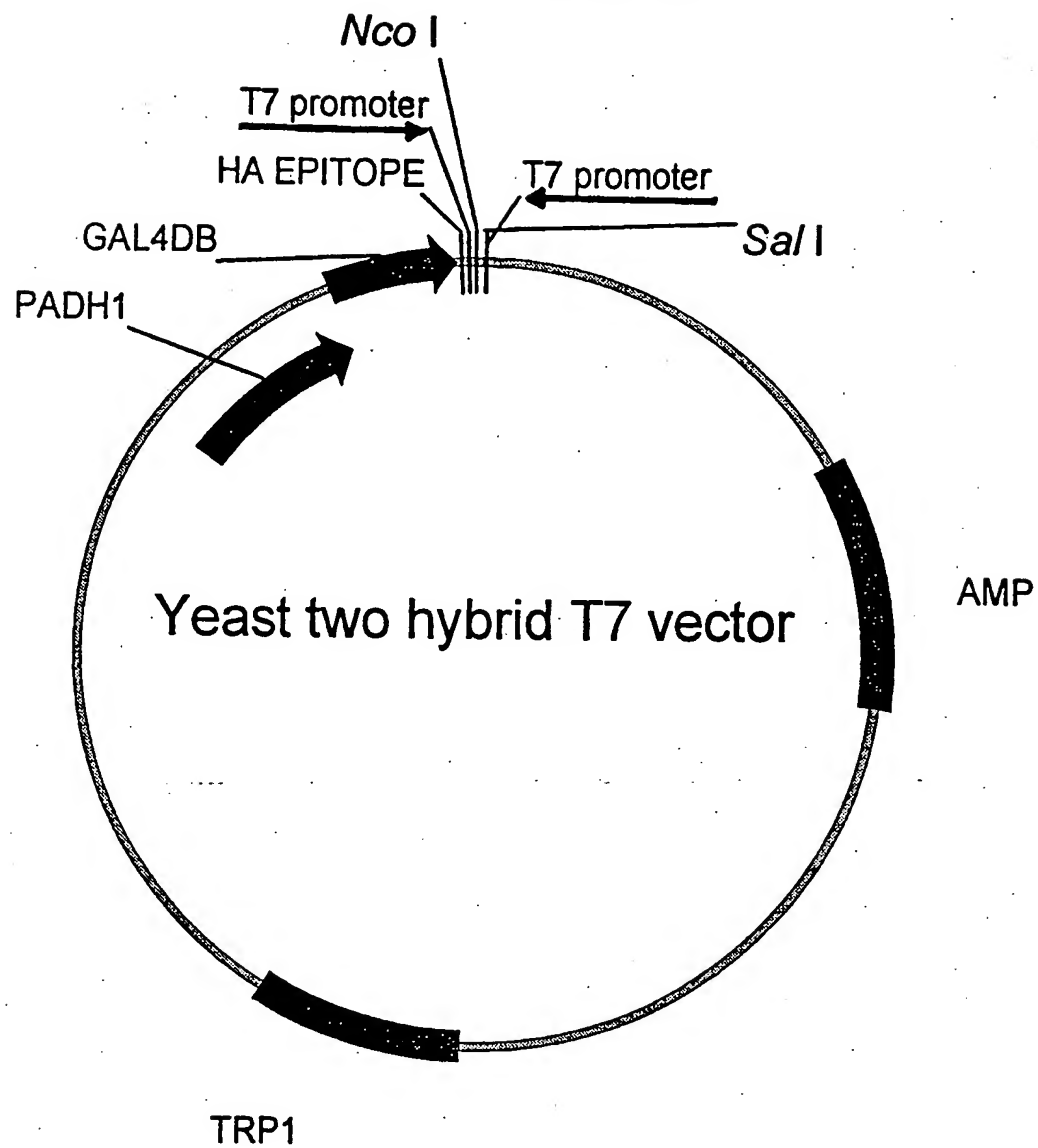
FIG. 2. (CONTINUED 2)

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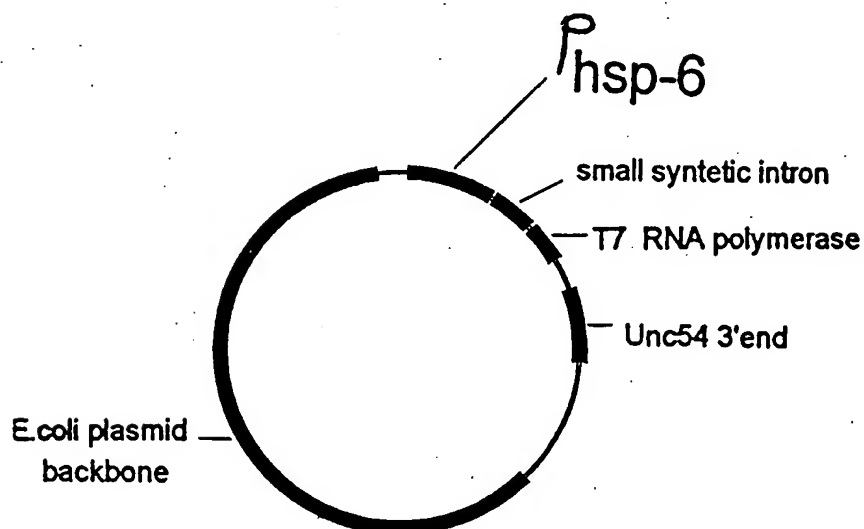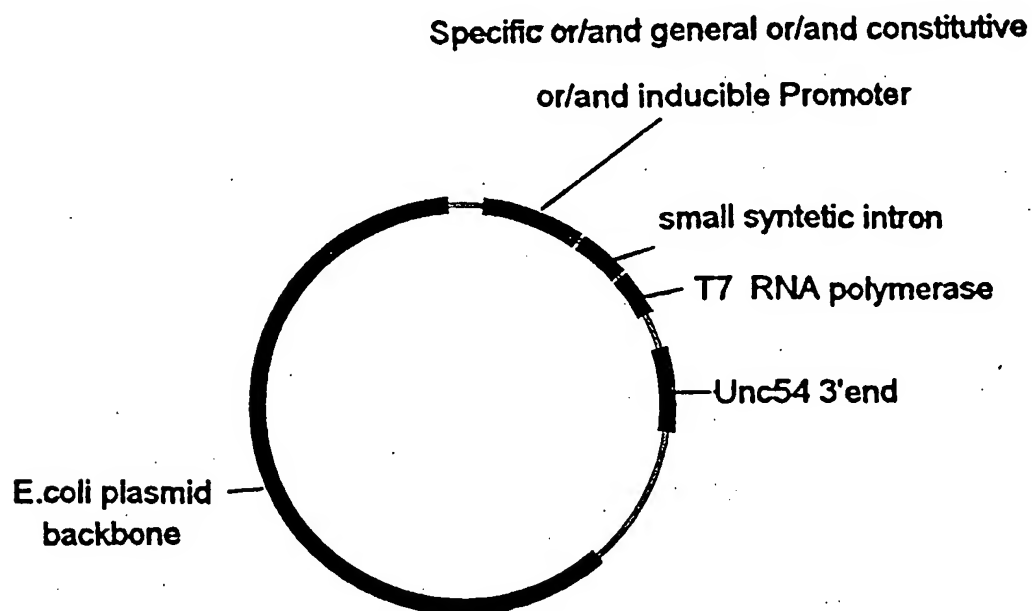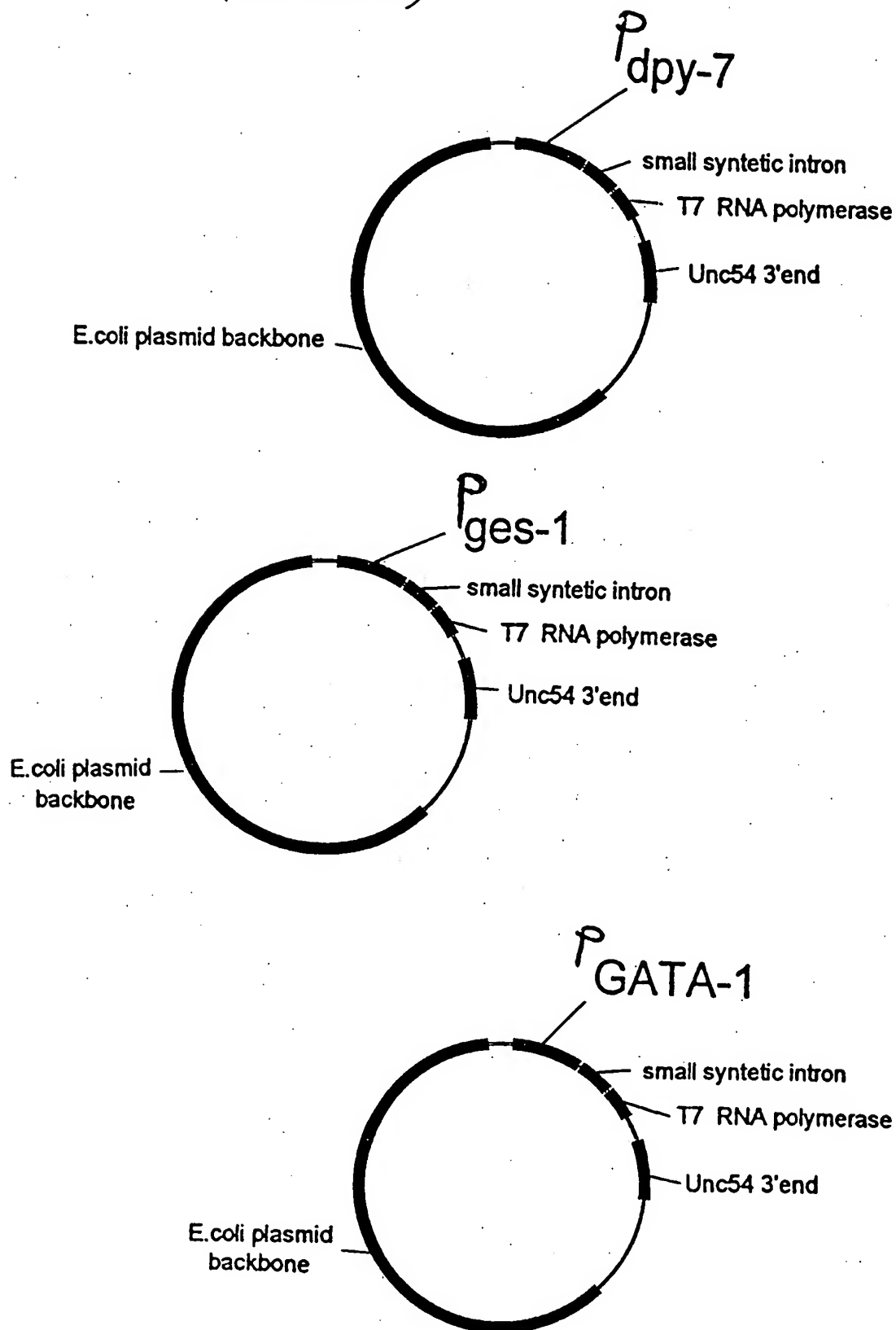cccgggattggccaaaggacccaaaggtatgtttcgaatgatactaacataacatagaacattttcaggaggacccttgg

FIG. 3

FIG. 4.

FIG. 5.

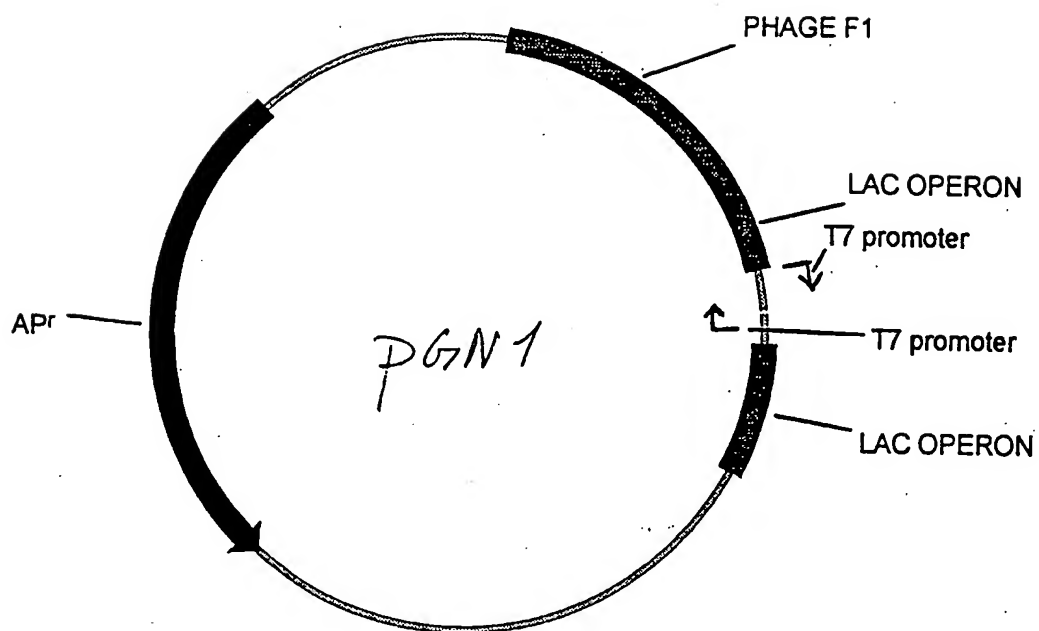General description of the C.elegans T7 RNA polymerase
expression vector with 4 examples

Specific or/and general or/and constitutive

or/and inducible Promoter

small syntetic intron

T7 RNA polymerase

Unc54 3'end

E.coli plasmid
backbone

P_{hsp-6}

small syntetic intron

T7 RNA polymerase

Unc54 3'end

E.coli plasmid
backbone

FIG. 5. (CONTINUED)

P_{dpy-7}

small syntetic intron

T7 RNA polymerase

Unc54 3'end

E.coli plasmid backbone

P_{ges-1}

small syntetic intron

T7 RNA polymerase

Unc54 3'end

E.coli plasmid backbone

P_{GATA-1}

small syntetic intron

T7 RNA polymerase

Unc54 3'end

E.coli plasmid backbone

FIG. 6.

PHAGE F1

LAC OPERON

T7 promoter

APr

PGN1

T7 promoter

LAC OPERON

FIG. 7

enhanced vector for RNAi, producing
sup35 ds RNA and ds RNA of the library,
gene of interest or PCR product.
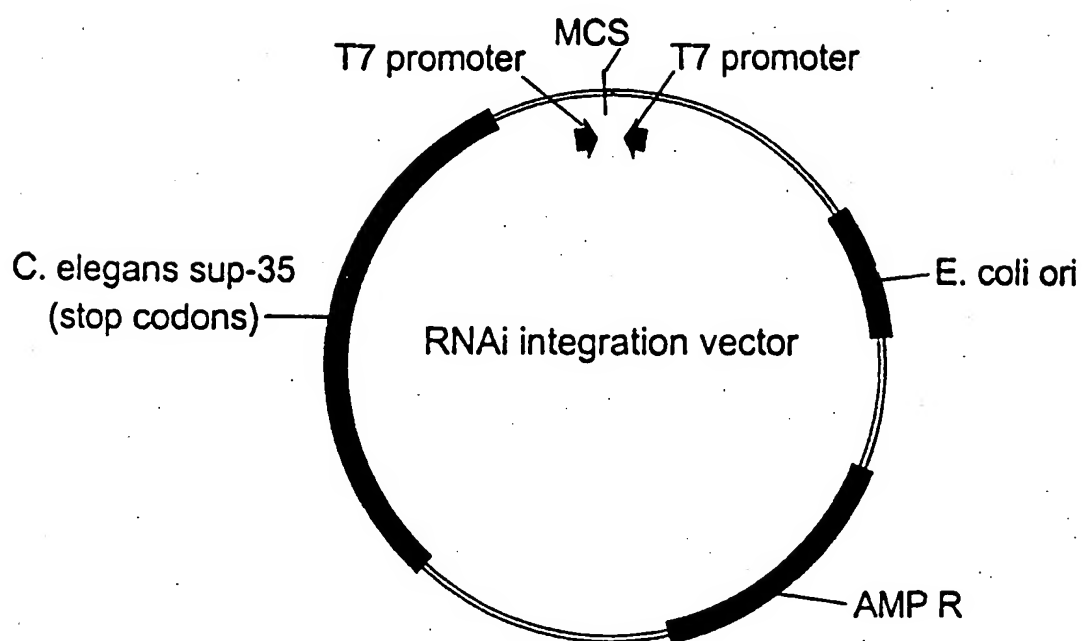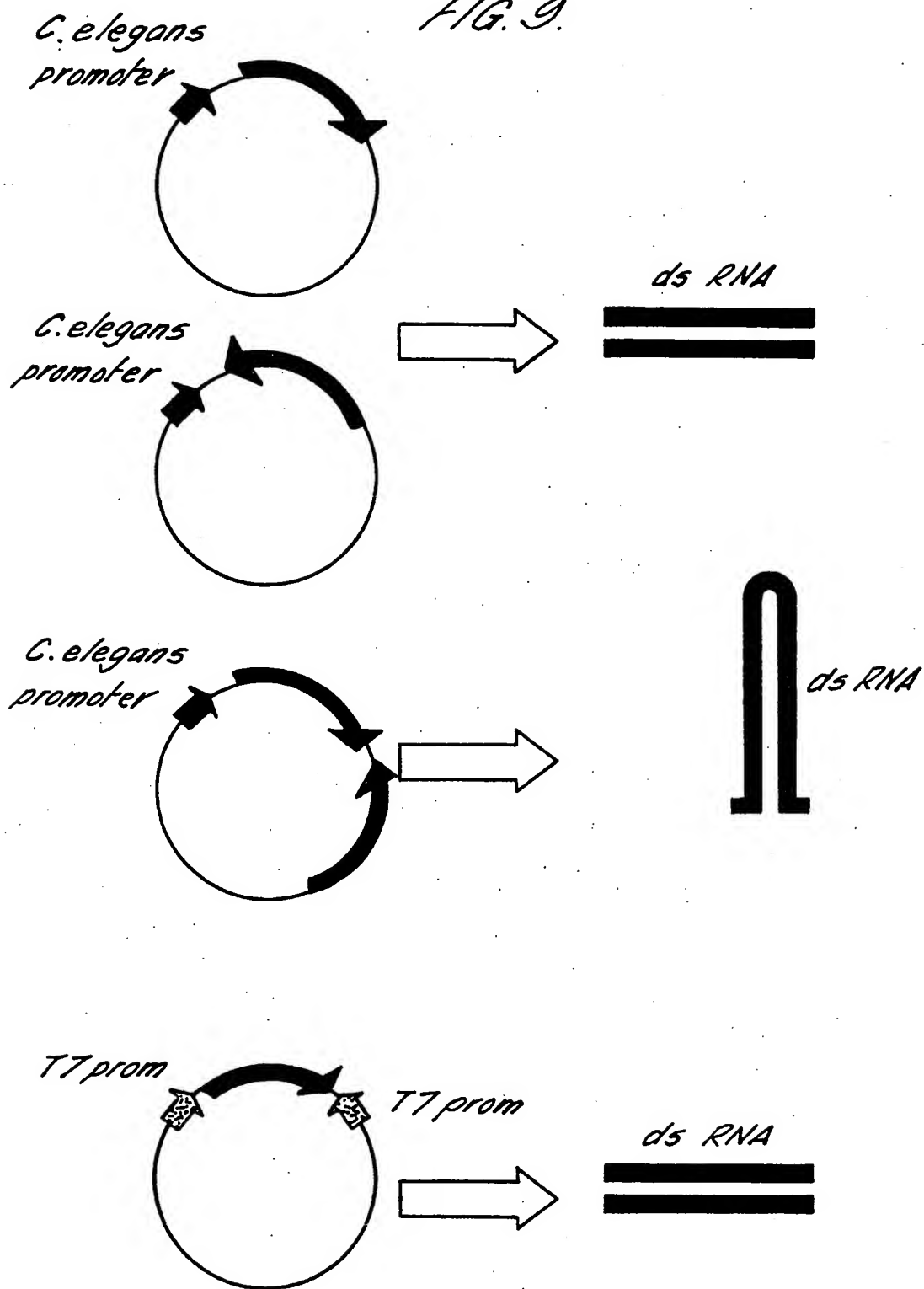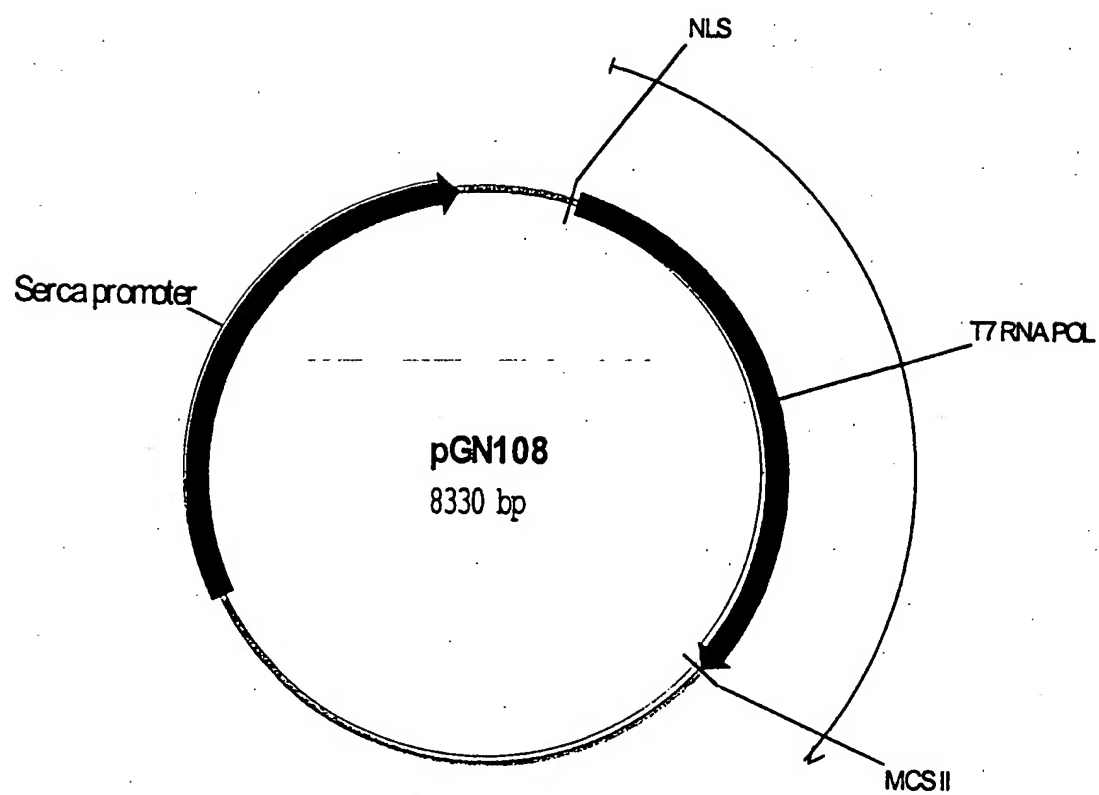
FIG. 8.

MCS

T7 promoter   T7 promoter

C. elegans sup-35
(stop codons)   E. coli ori

RNAi integration vector

AMP R

FIG. 9.

C. elegans promoter

C. elegans promoter → ds RNA

C. elegans promoter → ds RNA

T7 prom T7 prom → ds RNA

FIG. 10.

FIG. 11.

FIG. 12.

pGN400
4689 bp

MCS I
synth. intron
Acc 65I (282)
Sac I (292)
Xma I (293)
Age I (317)
T7-promoter
GFP
Eco R I (1208)
MSC II
amp

FIG. 13.

T7-promoter

pGN401
5175 bp

i
int1
ii
int2
iii
int3
iv
GFP
polyA signal
MCS II

FIG. 14.

FIG. 15.

pAS2* with Forward and Reverse T7/T3/Sp6

T7/T3/Sp6 promoter (forward)

T7/T3/Sp6 promoter (reverse)

GAL4DB

pADH1

7209 bp

AMP
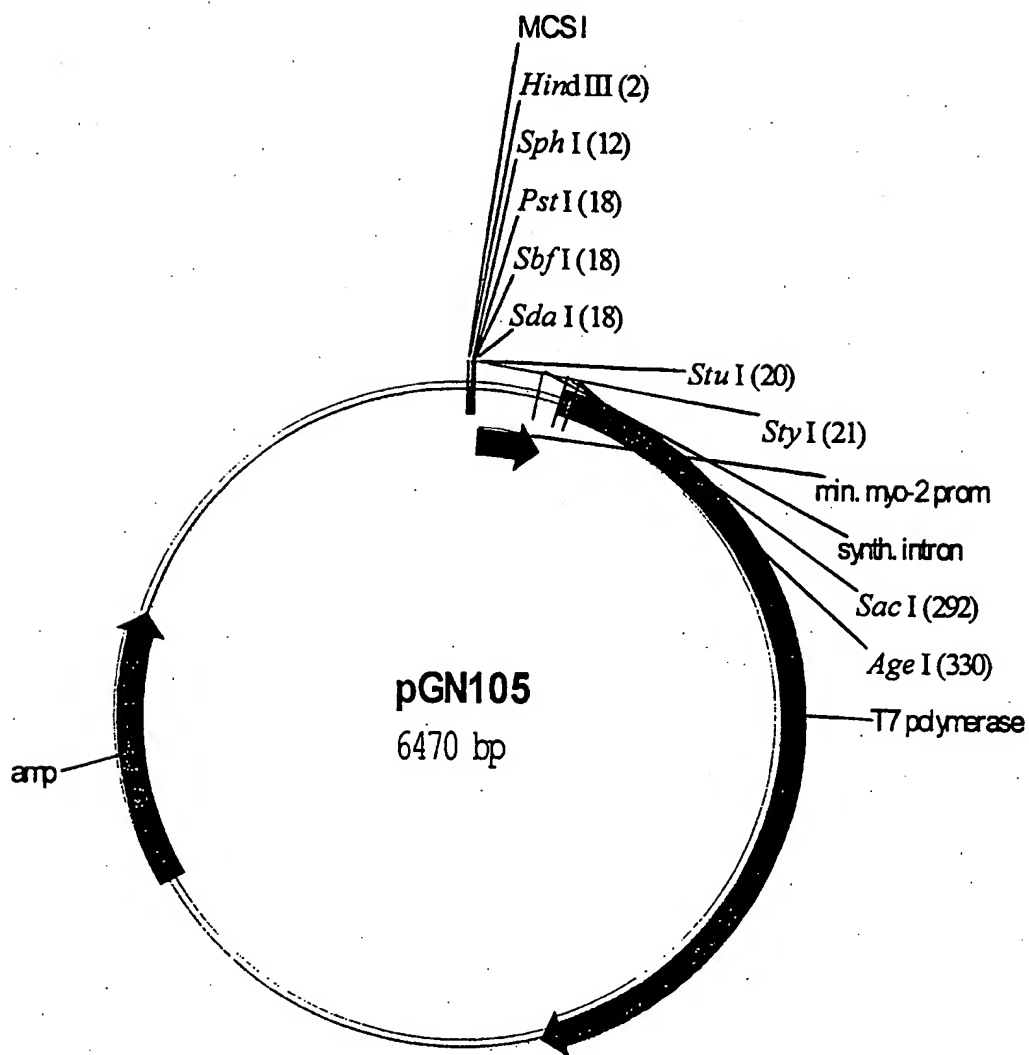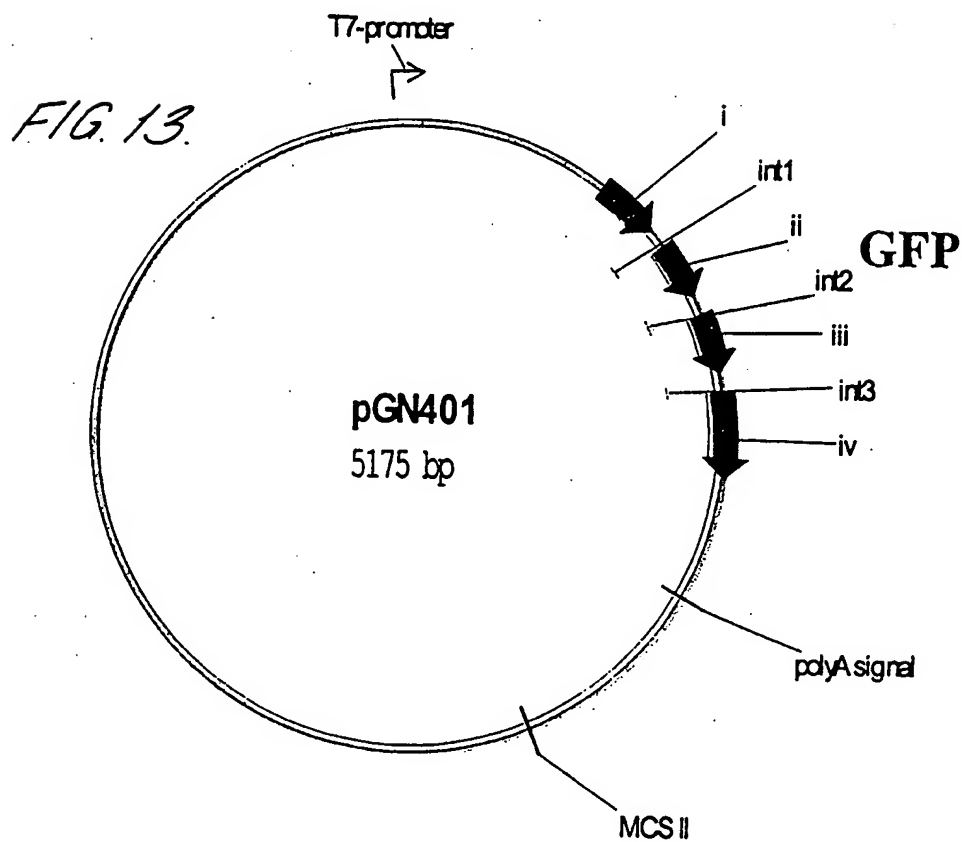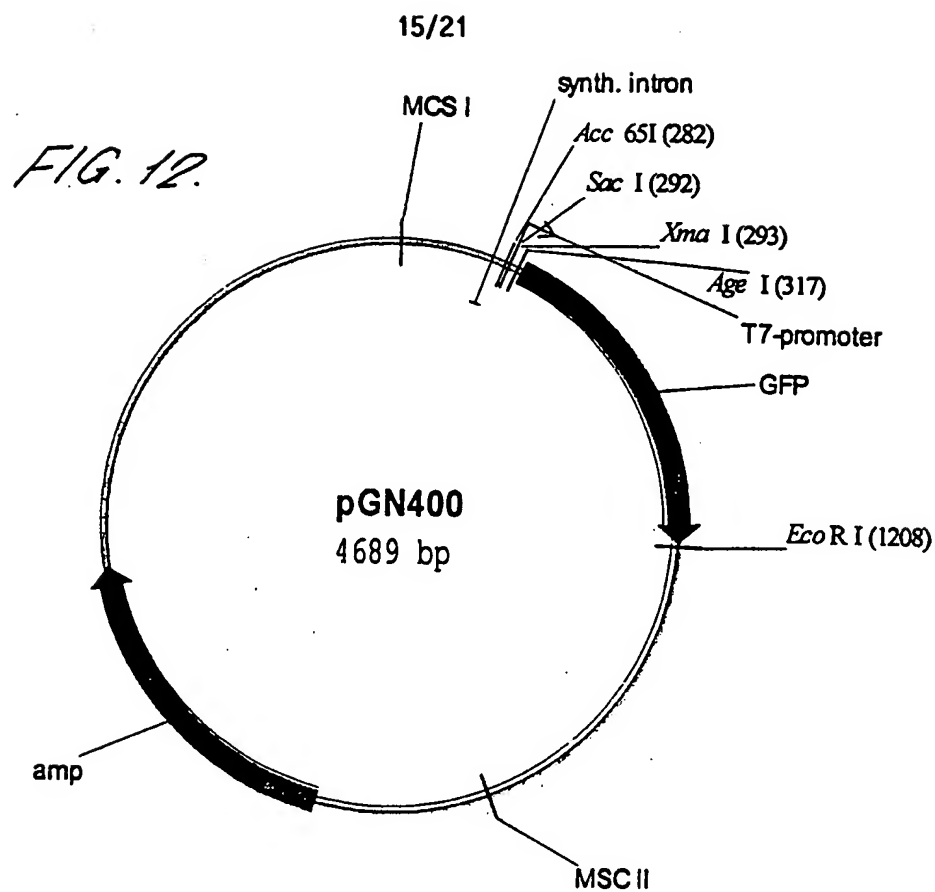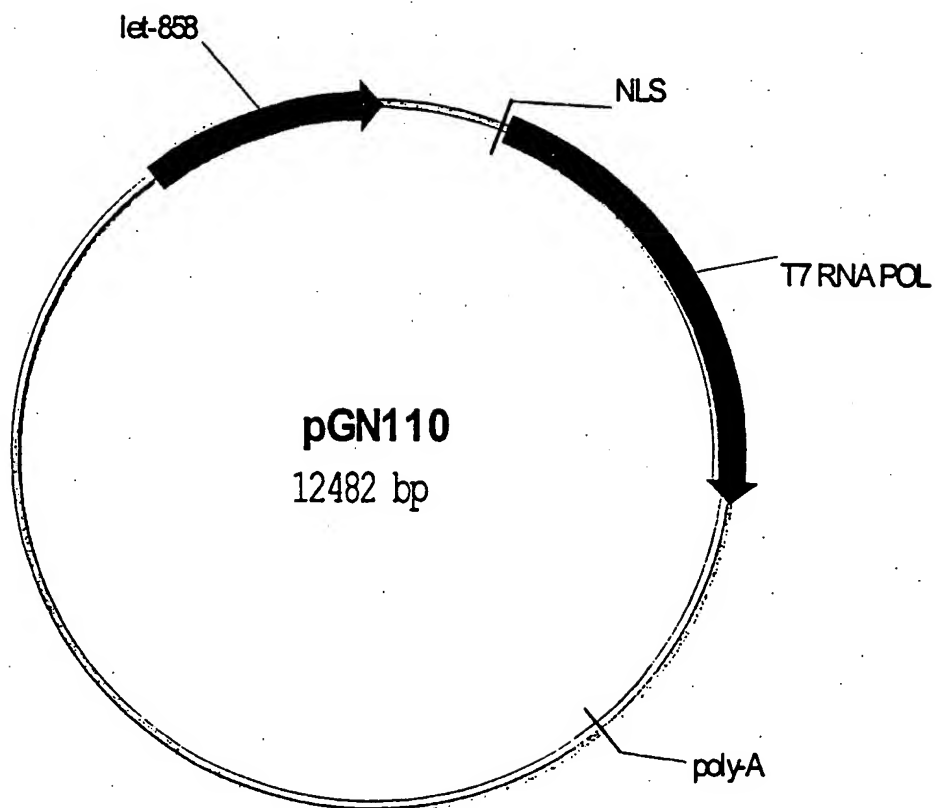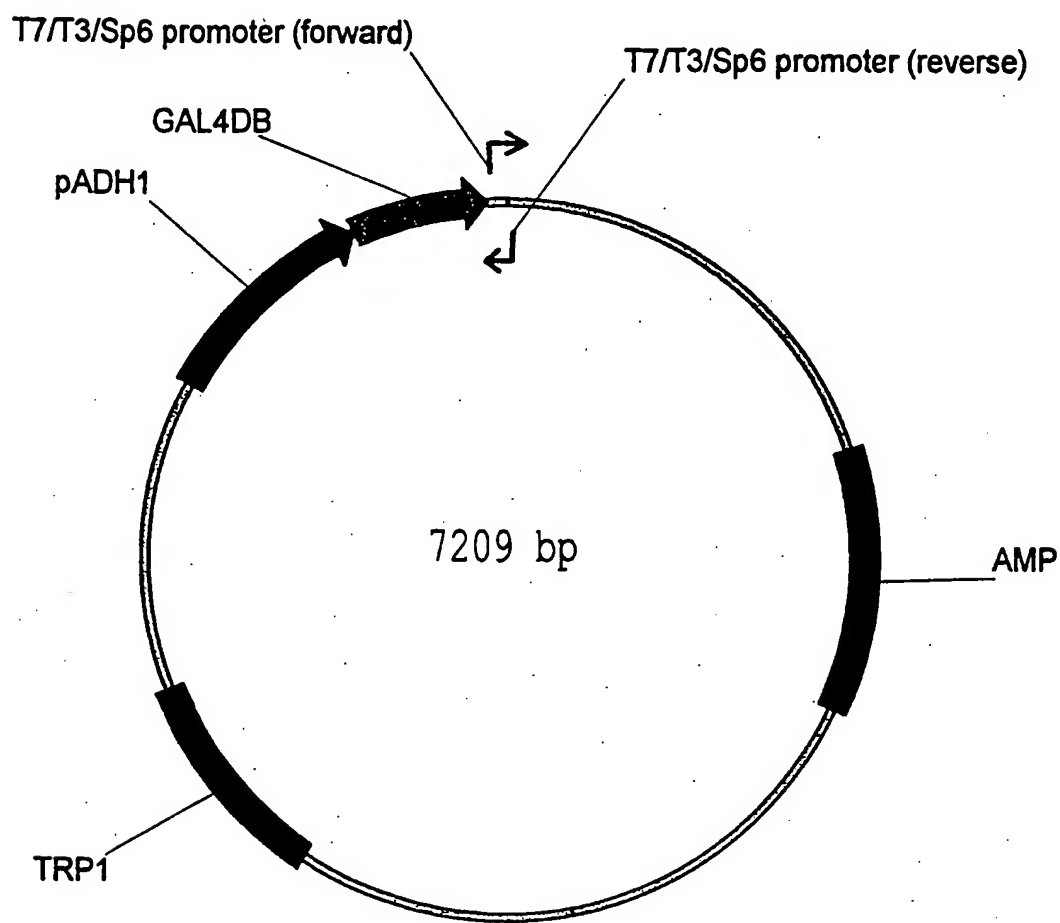
TRP1

FIG. 16.

pGAD424 with Forward and Reverse T7/T3/Sp6

FIG. 17.

FIG. 18.

T7/T3/Sp6 promotor
T7/T3/Sp6 promotor
Nco I (6704)
Apa I (6704)
Bsp 120I (6700)
*Eco*R I (6676)
Mlu I (6569)
GAL4AD
Kpn I (6326)
Acc 65I (6322)
ADH1
Nsi I (5557)
Ppu 10I (5553)
2MU-O
Eco 105I (5201)

*Bam*H I (1)
Sal I (7)
Pst I (43)
Msc I (181)
ADH1T
Bsr GI (699)
Eco RV (1074)
LEU2
Afl II (1505)
Cla I (1670)
Bst EII (1785)

pAD424-without-FULL-ICE-BOTH-7
6820 bp

Aat II (4427)
Sca I (3985)
Pvu I (3875)
AMP

Pvu II (2436)
ORI

FIG. 19